FROZEN BLOOD

Thesis presented for the degree of Doctor of Medicine

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by

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NEW

EXPERIMENTS

AND

OBSERVATIONS

TOUCHING

COLD,

OR,

An *Experimental Hiſtory* of *Cold*, begun.

To which are added

An *EXAMEN* of *ANTIPERISTASIS*,

And an *EXAMEN* of Mr. *Hobbs*'s Doctrine about *Cold*.

Whereunto is annexed *An Account of Freezing*, brought in to the *Royal Society*, by the learned Dr. *C. Merret*, a *Fellow* of it.

Together with an *Appendix*, containing ſome promiſcuous *Experiments* and *Obſervations* relating to the precedent Hiſtory of *Cold*.

Non legendum, ut exoganiendum, ſed inveniendum, quid natura faciat, aut ferat, Bacon.

By the Honourable *ROBERT BOYLE*,
Fellow of the *ROYAL SOCIETY*.

LONDON.
Printed for *Richard Davis*, Bookſeller in *Oxford*, 1683.

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THE LIBRARIAN AND STAFF, Glasgow University, have my thanks for help in locating original manuscripts, especially the Treatises by Robert Boyle in the Rare Books Collection. I enjoyed the rare privilege of reading original editions of his works, especially his description of Blood freezing. I felt a deep sense of history on these occasions.

To the thousands of volunteer blood donors I owe an immense debt for the trust they have placed in me to ensure that their donations are properly applied for the good of others.

To any whose names have been omitted, I apologise and express to them my sincere thanks.

RUTHVEN MITCHELL.

APRIL, 1976.

DECLARATION

I certify that the work contained in this thesis is entirely and wholly composed and presented by me, that the statements and assertions are entirely my own and that proper acknowledgement has been given to the work of others where appropriate.

.....Ruthven Mitchell. 5/4/76.

Ruthven Mitchell, B.Sc., M.B., Ch.B., M.R.C.Path
Deputy Director

PERMISSION TO COPY

SUMMARY

Cryopreservation techniques for the long term storage of human erythrocytes are no longer limited to research laboratories but are now within the technical capabilities of many larger Transfusion Laboratories as part of routine blood banking. Reconstituted frozen blood has been used successfully in massive transfusions, for combating shock, in open heart surgery, for rapid transfusion of hypovolaemic anaemic patients, for long term maintenance in aplastic anaemia and for exchange and intrauterine transfusions. This Thesis describes the history of this branch of cryobiology from its beginning in the 1950's until techniques were developed for preservation and viable recovery in the 1960's until today in the 1970's such techniques are well advanced.

The advances in technique described in this Thesis represent a period of five consecutive years of study and application. Descriptions are given of in vitro and later in vivo work with emphasis on the problems and solutions to the introduction and operation of banks of frozen red cells in the West of Scotland and the contributions made to the United Kingdom capability. Parts of this work have been delivered as scientific contributions at United Kingdom and International meetings of learned Societies. A list of these achievements is given and copies of reprinted scientific papers in the Appendix.

To allow detailed discussion, the text is, in places, repetitive but, I believe, only in this manner is it possible to fully comprehend the observations which have been made in each section. It did not prove easy to gather together numerous isolated reports scattered in the literature which on a number of occasions have been wrongly cited by others as to volume, page, title and even journal! Nevertheless I believe I have read and carefully assessed this subject.

The present author joined the staff of the West of Scotland Blood Transfusion Centre in September 1968. At that time, the Regional Director, Dr. J. Wallace had been contemplating the possible uses of a bank of frozen red cells in the Region and hence the author's interest in this field when he was invited by the Regional Director to pursue this subject as of special interest. As a result an application was made to the Scottish Home and Health Department for a special grant in aid amounting to £3,000 to carry out a feasibility study into the introduction and operating of a frozen cell bank in West Scotland. This application is detailed in the appendix and in July 1970 a new medical developments grant was awarded. In the interval the author had made a number of preliminary visits to centres already engaged in this work and certain contacts were made in the field of cryogenics with various commercial firms. In particular, reports were prepared of visits to the Netherlands Blood Transfusion Centre in Amsterdam and Regional Transfusion Centres at Brentwood, Bristol, and Army Blood Supply Depot, Aldershot as well as Guy's Hospital in London. All of these centres had shown interests in this field especially the Amsterdam, Army Blood Supply Depot and Brentwood workers. Accounts of these visits were prepared for the Scottish National Blood Transfusion Association to further the case for the introduction of a frozen cell bank. These reports are also included in the appendix together with an analysis of a visit to the British Oxygen Company who supplied most of our special requirements for cryogenic equipment.

By the end of 1970 we were ready to begin, and preliminary observations were made culminating in the successful transfusion of the first patient on 8th November, 1971. After this, progress was rapid and the author delivered a number of scientific papers in this field to the Low Temperature Society, G.B. (London November 1971) and to the West of Scotland Blood Club (May 1972) in order to gain support of clinical colleagues and to report progress. The first of these papers formed the basis for publication in J. Clin. Path. and is included in the appendix. Shortly after the methods had been introduced and made to work successfully the author had discussions with Professor Arthur Kennedy in the Renal Diseases Unit, Glasgow Royal Infirmary with a view to offering frozen recovered and processed cells to patients in the renal dialysis units in Glasgow. A later meeting was held at which haematologists and physicians were present to agree on the proposals put forward. Accounts of these meetings are given in the appendix. As a result, the first patient in a dialysis unit received frozen blood on 9th December, 1971. A request for blood for a dialysis patient in Dundee was met from the frozen cell bank in early January 1972. Sufficient cases had been treated by early 1972 such that the author presented a paper at the West of Scotland Blood Club. After this he was approached by Dr. M. Willoughby at the Sick Children's Hospital to provide frozen cells for dialysis patients and children requiring multiple intermittent blood transfusions.

At the end of a further year it was possible to take a decision about extending the frozen cell bank to include a satellite bank at Glasgow Western Infirmary where renal

transplantation requirements made a more accessible stock of frozen cells desirable. Meetings took place in October 1972, and a report prepared on the peculiar requirements of setting up a frozen cell bank on this location. A report of this is given in the appendix. As a result a bank was established in February 1973 - one year after the introduction of the principal bank at the Regional Centre. This marked the beginning of phase 2 in the present work and the problems encountered are dealt with in the main Thesis. This successful addition, naturally, led to the desirability of further extension of the frozen cell capability with proposals to have a satellite bank of frozen cells at Glasgow Royal Infirmary. This too, has now been achieved and discussion have taken place with Dr. J. Davidson of the Royal Infirmary to maintain uniformity of techniques. At the time of writing this, phase 3 has been successfully launched and technical staff have attended the Regional Transfusion Centre for special training in the methods required.

Throughout these developments numerous observations have been made by in vitro and in vivo study of frozen processed cells. These are described in detail. Additional logistical problems have been dealt with and new techniques introduced including a method of thawing recovery which formed part of another paper to J. Clin. Path. This is also included in the appendix of published works. In May 1974 a progress report was delivered as a paper to the West of Scotland Blood Club.

In April 1974 I attended a two day symposium of frozen cells held at the Army Blood Supply Depot, Aldershot where I delivered three papers on our experiences with frozen blood. Results of some studies have been reported by other colleagues at Haematology and Technical meetings.

The Scottish Home and Health Department have been most generous in allowing me to undertake various visits at home and abroad in connection with this work and to attending scientific meetings of the British Society for Low Temperature Biology of which I was elected a member in October 1970, six years after its foundation. Reports on these visits were prepared and these are included in the appendix.

Many other transfusionists in this country and abroad have visited our centre to study our methods which are set out in the appendix. This has led to similar developments in Aberdeen, Inverness, Guy's Hospital London and Leeds. Many consultants from most of the major transfusion centres in Britain attended a one day meeting on Frozen Blood organised by Dr. J. Jenkins and Dr. W. d'A Maycock on October, 3rd 1974 at the London Hospital. I delivered three papers to this meeting, entitled "Indications for the Use of Frozen Cells", "Organisation of a Regional Frozen Cell Bank" and "Organisation of a Hospital Frozen Cell Bank". Part of these papers are included in the appendix where detailed technical reports prepared by me are collected together.

This thesis is in two volumes. Volume I deals with the review of the discovery and methods of preventing freeze thaw injury in red cells and their development from techniques for preservation of small quantities to whole donations of blood. Later the problems associated with introduction of frozen cell banking are discussed and their solutions in West Scotland. Biophysical in vitro and in vivo measurements made throughout the study are given together with the effect of increasing usage of frozen blood in various clinical situations in this Region, other parts of Scotland and in a

World setting. The problems of logistical support for such clinical application are described in Volume II as well as the technical methods and materials used during the work and my various reports and publications based on my study and understanding of the problems of frozen cell banking. Extensive references are included in this volume.

Lastly it is a great pleasure to acknowledge the ready help and encouragement generously given by so many colleagues and friends listed in the Acknowledgement Section.

TECHNICAL REPORTS AND HISTORY OF DEVELOPMENTS

24:02:70	Visited British Army Blood Supply Depot, Aldershot. Letter to Major Robson.
27:03:70	Application for Development Grant to Scottish Home and Health Department. "Prolonged Preservation of Blood at very low temperatures" presented as part of the application.
10:07:70	New Medical Developments Grant from Scottish Home and Health Department approved - £3,000 plus maintenance costs.
7-8:10:70	Open day at British Oxygen Company. Report on visit detailing availability and types of equipment needed.
	Author elected member of Society for Low Temperature Biology.
20-22:4:71	Visit to Netherlands Red Cross Blood Transfusion Centre, Amsterdam. Report on visit.
May 1971	Interim Report prepared on progress.
7:05:71	First units of blood frozen.
5:11:71	Meeting with Professor Kennedy, Glasgow Royal Infirmary.
25:11:71	Further meeting at Glasgow Royal Infirmary with other physicians from dialysis units.
Nov. 1971	Scientific paper to Annual meeting of Society for Low Temperature Biology, London. Published in J. Clin. Path. "Storage and Retrieval and Inventory Control of Blood Donations and sub-samples kept in Liquid Nitrogen."
9:12:71	First request for frozen cells for dialysis patient.
May 1972	Scientific paper to Glasgow Blood Club, "Long term Preservation and uses of frozen cells".
July 1972	"Safety Notes and Methods", prepared for additional staff training.
19:10:72	Meeting at Glasgow Western Infirmary to extend into phase II.
Feb. 1973	Special meeting at Brentwood Regional Transfusion Centre on cryostable bags.
11-12:10:73	Annual meeting of Society for Low Temperature Biology Report.
3-4:04:74	Special meeting at Army Blood Supply Depot, Aldershot. Author gave three scientific papers.

TABLE 1/1

4-8:09:74	11th Annual Meeting of the Society for Cryobiology jointly with Society for Low Temperature Biology, London. Report on meeting.
3:10:74	Special meeting at the London Hospital on Frozen Blood. Author gave three scientific papers.

TABLE 1/1 (Cont'd)

PART I

HISTORY AND DEVELOPMENT OF METHODS OF FREEZING BLOOD

'The third day comes a frost,
a killing frost'

Shakespeare; Henry VIII

A pint of Sheeps blood did freez at the top, and all the ſides of the diſh wherein 'twas put, and was nothing elſe but the *ſerum* of the blood. This ice being ſeparated from the blood, and thaw'd at the fire, and then again expoſed, congealed into a ſeeming membranous ſubſtance, and was taken for ſuch by ſome that ſaw it, and ſo continued in a warm ſeaſon, and appeared in all reſpects a membrane. This alſo was ſeen and regiſtred in the Journal. The blood remaining gave me no ſigns that froſt had taken it.

A pint of Sheeps blood did freez at the top, and all the sides of the dish wherein 'twas put, and was nothing else but the serum of the blood. This ice being separated from the blood and thaw'd at the fire, and then again exposed, congealed into a seeming membranous substance, and was taken for such by some that saw it, and so continued in a warm season, and appeared in all respects a membrane. This also was seen and registered in the Journal. The blood remaining gave me no signs that frost had taken it.

Plate 1/7. Robert Boyle's Description of Blood Freezing 1683

MEMOIRS FOR THE NATURAL HISTORY OF Humane Blood,

Especially

The Spirit of that Liquor.

By the Honourable

ROBERT BOYLE

Fellow of the Royal Society.

Etsi enim haud pauca, eâq; ex præcipuis, supersint à solvenda, tamen consilium & vinctum opus potius promovere in multis, quàm perficere in paucis. Verulam. in Præfat. ad Histor. Natural. & Experiment.

LONDON,

Printed for *Samuel Smith* at the *Princes Arms* in St. *Pauls Church-yard*, 1683/4.

were prettily shaped, being comprehended by Planes smooth, finely figur'd, and aptly terminating in solid Angles, as if the Concretions had been cut and polished by a Jeweller.

To the same Title,

Experiment 6.

THere is another way that I have used to observe the Figures of the Salt of Blood which was to rectify the Spirit of Blood, so as it may be fully satiated with the Salt, whilst the Liquor (in the Receiver) continued yet somewhat warm. For then setting aside this over impregnated Liquor when it came to be quite refrigerated (which should be done very slowly) there appear'd at the bottom of the Vial a good number of Saline concretions of differing Sizes,

Sizes, several of which, as far as the rest would suffer me to see them, were shot into Crystalline Plates very smooth, and prettily figur'd, having to the best of my conjecture, their broad and parallel Surfaces of a Hexagonal or an Octogonal Figure regular enough.

To the same Title,

Experiment 7.

ACcording to the *Hypothesis* of divers Learned Naturalists and Physicians, I suppos'd it would be thought considerable, to know what would happen upon putting together the Volatile Salt of Humane Blood, and the Spirit of Nitre, with the more fugitive parts of which Salt they conceive the Air to be plentifully, and some of them to be vitally impregnated.

To

AN EARLY EXPERIMENT IN CRYOBIOLOGY ?

Plate 1.3

HISTORY AND REVIEW OF LITERATURE

(Plates 1/2 & 1/3).

300 years have passed since Robert Boyle published a monograph entitled 'New Experiments and Observations Touching Cold' in which he discussed experiments on cold storage of fruit, eggs and meat together with their changes in texture after freezing and thawing. Living frogs and fish would survive short periods of encasement in ice but not complete body freezing. He recorded that corpses buried in the Greenland snow were recovered up to 30 years later in a perfect state of preservation with rapid dissolution on thawing. During the 18th and 19th centuries others including Reaumur, Leeuwenhoek, Spallanzani, John Hunter and Claude Bernard studied the effects of low temperature on living organisms. Most were attracted by the idea that it might be possible to preserve whole plants and animals and even whole human beings in a state of suspended animation by cooling to temperature low enough to arrest both vital and corruptive processes. Smith (1970), Boyle (1683), Pouchet (1866).

Numerous publications in cryobiology literature deal with a wide variety of living matter - zoological, botanical, including unicellular and multicellular systems. In 1961 Smith reviewed the biological effects of low temperature with particular reference to work since the second World War. Even then she was unable to cover all biological systems. There is still considerable interest in the problems of man in a cold hostile environment and the ways in which plants, animals and man can survive the climatic rigours of high altitude, space flight and the problems of adaptation in

polar lands and seas. There are now two societies devoted to Cryobiology. The American Society formed in 1964 and the British Low Temperature Society in 1965, of which the author was elected a member in 1970.

B.J. Luyet (1949 a & b) is credited as having first reported preservation of erythrocytes by rapid freezing by immersion of thin films of blood in liquid nitrogen at -196°C. No protectant was added and it has since been appreciated that the reason for 'preservation' was that the rate of cooling was so rapid as to prevent high salt concentration within the cells which would otherwise cause cell destruction (Pegg, 1970; Farrant, 1970). Such a method was therefore fortuitous but modern methods using larger volumes of blood and modern long term storage banking methods rely on this observation, which is applicable only to red cells which are uniquely different from mammalian nucleated cells in that water permeability of red cells is so high whereas in nucleated cells the permeability is much lower and allows ice crystal formation within the cell thus permitting hypertonic salt damage to occur. In such cases cellular penetrating neutral solutes to minimise the rize in salt concentration are used (Florio, Stewart and Mugrage, 1943; Doebbler and Rinfret, 1962; Rinfret, 1963).

These substances are similarly used in the preservation of large bulk volumes (such as whole donations) of human red blood corpuscles, the concentration of neutral solute determining the optimum rate of cooling. Practical methods of banking donations of blood have been developed for laboratory

and clinical use whilst maintaining sterility. This review will trace the history behind these discoveries and show how they have been adopted and adapted to achieve further refinement with significant observations on the use of frozen blood in patient care. (Bishop, 1970, Spielman and Seidl, 1970)

Several excellent reviews of the earlier literature on cryopreservation of cells have appeared (Smith 1961, 1970; Turner, 1970). Turner (1970) indicated that his task had been sponsored by the office of Naval Research in view of the increasing complexity of the subject and the large amount of interest being shown in freeze preservation. Clearly military stockpiling of donor blood had a strategic usefulness and therefore he performed a thorough search and evaluation of the literature reviewing some 400 separate papers and treatises. Since that time (1970) an ever increasing number of clinically useful papers have been published and methods can be divided into a number of broad categories reviewed later in this work.

In 1949 Polge et al accidentally discovered that addition of 5-20% glycerol to semen would protect fowl and human spermatozoa against the lethal effects of freezing to -79°C. Previous published work by Luyet and Hartnung (1941), and Rostand (1949) had hinted at such an effect. Polge found that propylene glycol, ethylene glycol ('anti-freeze') and other related polyhydric alcohol homologues were more toxic than glycerol but later work in 1954 by Lovelock showed that a variety of monohydric, dihydric and polyhydric alcohols, amides and sugars, including methanol, acetamide and glycerol monoacetate could give successful cryopreservation. Sperm

Sperm from non ovine and non human sources were not protected in Polge's experiments and since some shrinkage of the acrosomal cap appeared it was concluded that dehydration had occurred.

In 1950 Smith investigated the effect of various concentrations of glycerol on erythrocytes which in effect behaved as osmometers. Surprisingly human cells registered little distortion and change even when suspended in solutions of 10 to 15% glycerol. Clearly the erythrocyte membrane must be permeable to glycerol and freeze sensitive spermatozoa must be impermeable. She showed that red cells frozen in glycerol could be recovered intact even with freeze temperatures down to -79°C with optimal glycerol of 10-15%. Storage at -79°C could be maintained for up to six months after which red cells could be recovered apparently intact. Smith therefore showed that rabbit and human erythrocytes could be suspended in 1.4-2.0 M glycerol which rapidly established an equilibrium between the internal and external concentration of glycerol such that these cells would then survive freezing to -79°C and subsequent thawing.. This recovery was dependent upon the speed at which the cells returned to a glycerol free isotonic medium, with the best recovery after slow dialysis and worst recovery after rapid 'deglycerolization'. Lysis in isotonic media was due to the more rapid diffusion of water into the cell due to the hygroscopic properties of glycerol resulting in cell distension and rupture (endosmosis). Willis (1965) much later stated that the rate of water exchange is four fold greater than glycerol across the red cell membrane. Hence

the principle underlying the recovery of cells by using non-penetrating or slowly penetrating sugars and macromolecules to bind water outside the cells until the slower moving glycerol can be exchanged from a high intracellular to low extracellular concentration. For each reduction in cellular water content of about 20-30% there is a decrease of approximately 1-log in the rate of heat exchange necessary to avoid ice crystal formation. Thus a rate of 1000 large cal/sec is necessary to avoid ice formation when the temperature of an 80% pure water solution is lowered below freezing; a rate of 100 cal/sec must be exchanged for 60% pure water and 20 cal/sec for 30% pure water. Tullis (1965) cited the work of his colleague Pyle that if one takes the normal cell water to be 72g/100ml and whole blood as 83gm/100ml it would require 40-50% glycerol effectively to displace the aqueous phase down to a concentration of 20-30%. This is the actual amount found in practice in the "slow freeze" method where there is less attention needed to optimize rate of freezing and thawing, type of container and thermodynamic conditions (Pert et al, 1965). In order to speed up the removal of glycerol apart from improvements in equipment, one approach is to reduce the amount of glycerol added but increase the rate of cooling as recommended by Meryman (1972), Pert et al (1965) and Krijnen (1964). Pert et al (1965) found that above 40% glycerol high recoveries were obtained regardless of the freezing rate. With faster freeze rates lower concentration of glycerol could be used but then films of blood only could be frozen. Recovery of 98.1 + 0.2% was

obtained by freezing rates of 144°C/minute and glycerol concentration of 14% w/v. They showed that for any chosen glycerol addition there was an optimal rate of freezing expressed as $TC^{1.5} = 750$, where T is maximum freezing rate in °C/min and C is concentration of glycerol in vol %. At 20 vol % glycerol, a wide range of freezing rates could be used and, hence, with liquid nitrogen users, 14-20% glycerol concentration have all been adequate to ensure good recoveries, when the blood layer thickness being frozen is 4-10 mm. This forms the basis of the methods used by Krijnen et al (1965). As well as this protective effect of glycerol it was also evident that there was an optimum rate of cooling of the glycerolised cells. If freezing was prolonged so that it took 1 hour to reach -79°C the results were less satisfactory and poorer recoveries were achieved.

Parker (1951) reported experiences on freezing to -79°C of guinea pig and rabbit blood protected by glycerol with a final concentration of 10-15%. One hour after thawing the majority of red cells appear morphologically normal. Later experiments showed similar results after storage at -79°C for three months. Sloviter (1951, 1951a) reported the use of glycerol to prevent haemolysis in rabbit and human erythrocytes.

In 1951 Smith et al extended their observations to include a direct visual observation of the appearance of glycerolised red cells on the freezing stage of a microscope. The pattern of ice crystals formation showed that crystals were smaller in

glycerol media down to -40°C and that cells lay in columns between extracellular crystals. Thus grew up one of the theories of cryopreservation - that ice crystals expand as they form and coalesce, that such expansion causes physical rupture of cell membranes, that smaller less expansive, less numerous crystals form in the presence of glycerol which being hygroscopic retains water which would otherwise be available for crystal formation and accretion. Pouchet (1866) many years previously in his detailed study of freezing whole animals, considered that damage to red cells was due to sharp spicules of ice.

In 1953 Lovelock further expanded knowledge of the mode of action of glycerol and suggested that it behaved as a 'salt buffer' which prevented the haemolysis which occurred due to a rising salt concentration in the remaining fluid as water in the system separates out as ice. Lysis begins at a concentration of sodium chloride of 0.8M at about -30°C and diminishes again at temperatures below -40°C. The Addition of glycerol to the system showed that the critical temperature at which damage began and the extent of the damage at any given temperature both fell, until, in the presence of 2.5M glycerol, no haemolysis occurred at any temperature maintained for up to 10 minutes. The temperature at which lysis first occurred was that at which the remaining fluid phase became 0.8 molar in sodium chloride.

Farrant (1970) writing on the injuries sustained by living cells at low temperatures expresses these earlier findings in a modern biochemical and biophysical manner. The normal

concentration of sodium chloride in normal blood and tissue fluid is 0.15M (isotonic). Increasing the concentration of sodium chloride in a suspension of red blood cells has several effects. Between isotonic and 0.8M the cells are unharmed whereas at concentration of 2.0M or greater the cells undergo progressive haemolysis in an increasingly hostile environment. Between 0.8M and 2.0M the cells are damaged but this is not apparent. This latest damage can be revealed by two forms of challenge. One is to resuspend the cells in the original (0.15M) solution leading to hypotonic haemolysis due to swelling and bursting. The second stress is to cool the cells abruptly by 10°C or more. This 'thermal shock' leads to partial haemolysis due to damage to the lipoprotein membrane of the cells. During the process of freezing therefore the cells are exposed to both forms of stress - an increasing electrolyte concentration as water turns to ice, and thermal shock. During subsequent thawing ice is melting and salt concentrations are decreasing with consequent hypotonic haemolysis.

Although most reviewers consider Luyet (1949) to be the father of modern cryobiology, it is important for historical record to note that Doebbler et al (1966) and Farrant (1970) have established the contribution made in this field by Florio, Stewart and Mugrage (1943) and Woodcock, Thistle, Cook and Gibbons (1941). Much of the early work has been ignored or overlooked but when I read these papers I was convinced of their rightful place, especially that of Florio et al where descriptions are worthy of quotation, "since it seemed to us theoretically possible to preserve red blood cells by quick

freezing we were stimulated to study the response of the erythrocyte to this procedure". They described 97 - 98% preservation using cryoprotective sugars and cooling of spray droplets and small volumes of human dog, sheep and rabbit cells in carbondioxide and liquid air coolants. They reported that oxygen carrying capacity and antigenicity of recovered cells was unimpaired.

MECHANISMS OF CELL INJURY BY FREEZING AND THAWING

It is self evident that freezing any cell population is harmful. Before considering methods of preventing cell injury therefore it is necessary to study the mechanisms whereby cells are damaged or destroyed by cold. The usual effect of cold on living cells is to slow the metabolic (vital) activity and to slow the physical processes. In this way it is possible with the addition of suitable substrates and a means of removal of waste products to keep cells alive for up to a few weeks. For longer periods of time however it is necessary to use sub-zero temperatures and the problems of freeze-thaw injury must be investigated and if possible understood with a view to minimizing the effect. At -79°C (the temperature of solid carbon dioxide and some mechanical deep freezers) cells can be preserved for a long time whereas with liquid nitrogen refrigerant at -196°C the storage is probably indefinite. Even at this temperature however, since it is not absolute zero, some very low molecular activity is still possible and there might be an almost imperceptible metabolic run-down over many years. "Even the Pyramids wear away" as one author has put it.

The majority of living cells contain 75-80% of water (red cells - 60-67% - the "driest" cells in the body) and as freezing occurs the concentration of solutes in the extracellular liquid phase increases as ice comes out of solution and intracellular ice also forms.

The raised extracellular solute concentration especially of electrolytes is thought to damage the cell lipoprotein membrane and severe changes in pH and buffering occur both

inside and outside of the cell (Lovelock 1953 a,b, 1954). Obviously the quicker the rate of cooling the less time will the cell membrane be exposed to these stresses. But there is an optimum freeze rate (55°C/second for red cells) because at lower rates intracellular ice does not form. (Farrant, 1970). If these two effects overlap - a cool rate to cause hypertonic extracellular fluid and intracellular ice formation then no recovery is possible without some form of cryoprotective substance being present. Meryman (1971) in a review of the mechanism of freezing in red cells proposed the following.

The concentration of non-penetrating solutes through freezing leads to a reduction in total cell water and a reduction in size. When roughly 64% of the cell total water has been removed and the cell reduced to about 55% of its original volume, a resistance to further shrinkage develops leading to an osmotic pressure gradient across the membrane. Cells so stressed are susceptible to thermal shock or to a spontaneous increase in permeability for small molecules. During freezing, cells that resist the stress become increasingly susceptible to thermal shock and may haemolyse with continued temperature reduction. Cells which spontaneously succumb to the osmotic stress experience an influx of extracellular solute rendering them potentially hypertonic leading to immediate haemolysis on thawing. Glycerol and related cryoprotective agents reduce the proportion of ice found and thereby prevent the concentration of extracellular solutes, preventing the reduction of cell size beyond the tolerable minimum.

Human erythrocytes can be frozen to nearly -3°C without injury (Strumia. 1949) and on freezing to lower

temperatures an increasing proportion of cells are found to be haemolysed immediately following thawing. 100% haemolysis is seen following slow freezing to -10°C or below. Some animals can withstand the freezing out of a large portion of their water as extracellular ice whilst others including arctic mammals and fishes have adapted to cold in remarkable ways (Meryman 1971).

Damage due to High Concentration of Solutes

If red cells are suspended in hypertonic solutions between 0.8M and 2M no obvious harm results yet damage has occurred and can be demonstrated in two ways: firstly by osmotically stressing the cells by resuspension in normal isotonic media (0.15M sodium chloride). This causes swelling and hypotonic haemolysis, secondly by cooling the cells abruptly by 10°C or more causing thermal shock haemolysis. Sodium chloride concentrations greater than 0.8M damage the lipoprotein of the cell wall. In freezing cells 0.8M is reached at -3°C and 2.0M at -7°C. The cooling of cells at sub-zero temperatures therefore results in "thermal shock" and the thawing on subsequent recovery results in hypotonic stress. At temperatures below -3°C more than 82% of the water solidifies and the salt concentration in the residual liquid phase approaches 0.85M (Strumia, Colwell and Strumia 1960). At salt concentration of 0.85M or higher, the red cells become highly susceptable to thermal and osmotic environmental changes (Lovelock 1953 a,b, 1954) and denaturation of lipid protein complexes occurs (Lovelock 1957).

Damage due to Formation of Intracellular Ice

The amount of water remaining in the cells at any

temperature during sub-zero cooling will determine the probability of intracellular ice formation. Mazur (1963) has computed these factors and derived formulae to express the concept. In considering these Farrant (1970) was unable to explain the extremely complex changes which must occur inside cells in such conditions.

The mode of action of glycerol and other penetrative intracellular protectants has already been described but at this point one should reiterate that the main action is to lower the freezing point so that at any given temperature below that at which ice starts to separate there will be a higher proportion of fluid in the media which contain glycerol than in those that do not. Since the salts remain in the fluid phase until their eutectic point is reached, the natural consequence is that there will be a lower salt concentration at any specified temperature when glycerol is present, since although less water is available for ice formation, glycerol bound water can still act as a solvent for cations and electrolytes. The temperature at which lysis of red cells occurs is that at which the remaining fluid phase becomes 0.8M in sodium chloride. Hence glycerol is sometimes said to have a salt buffer effect. This is the reason that glycerol, Dimethyl sulphoxide and similar compounds reduce damage and allow low temperature storage of many types of salt-sensitive mammalian cells.

With non-penetrating low molecular weight substances (sucrose, dextrose etc.) there is a higher eutectic point and thus they do not have as good an effect as glycerol in reducing the volume of ice and the hypertonicity of

electrolytes during freezing. In practice these compounds are therefore less efficient in reducing salt damage. With non-penetrating high molecular weight substances (polyvinylpyrrolidone, glycol) it is thought that these protect the cell membrane by forming a protective coat to the cell membrane. (Farrant 1970)

It is important to distinguish between the cooling rate and the freezing rate. They are each concerned with the time required for the passage of water from the time the specimen is first cooled until it reaches the desired storage temperature. The cooling curve begins at the starting temperature of the material and continues until freezing begins, then begins again after the completion of freezing and continues to the lowest temperature reached. The freezing curve is the portion between the two cooling segments. Expressing the cooling and freezing temperatures against time gives velocity of cooling and freezing rates. The freezing curve is in three parts.

(a) the rapid initial propagation of ice when the liberation of heat by the ice in formation is in large excess over the heat withdrawn in cooling.

(b) the stationary phase (plateau) which represents a slowing of the pace of the propagation of ice and the heat liberated is balanced by the heat withdrawn.

(c) the advanced stage of freezing when the amount of heat withdrawn exceeds the gradually vanishing amount of heat liberated.

Obviously there are various factors which can influence these rates such as the rate of advancing of the freezing front, the rate of heat removal by the refrigerant and the rate of liberation of latent heat.

There is no universal agreement among cryobiologists

as to the limits to be set for these various rates. Thus one reads of slow, rapid and ultrarapid freezing rates. Rapatz and Luyet (1965) considered freezing at from $-5^{\circ}C$ to $-50^{\circ}C$ as being low cooling rate and cooling at $-80^{\circ}C$ to $130^{\circ}C$ as being high cooling rate. Krijnen et al (1966) considered that slow cooling rates were $10^{-3\circ}C$/sec, intermediate $1^{\circ}C$/sec, rapid $3^{\circ}C$/sec and ultra rapid 100 to $200^{\circ}C$/sec. Hurn (1968) considered slow cooling rates as 1 to $10^{\circ}C$/min, rapid 100 to $1000^{\circ}C$/min and ultra rapid more than $10,000^{\circ}C$/min. It is almost impossible to obtain such rapid cooling as $10,000^{\circ}C$/min in the physical world and such extensions of the definition do not help in trying to understand the difficulties encountered due to authors selecting a wide range of cooling rates (Doebbler et al 1966).

Rapatz and Luyet (1968) considered $-5^{\circ}C$ to $-20^{\circ}C$ as low cooling rate ($15^{\circ}C$/sec), $-30^{\circ}C$ to $-60^{\circ}C$ was intermediate ($35^{\circ}C$/sec) and $-70^{\circ}C$ to $-130^{\circ}C$ was high ($120^{\circ}C$/sec). Finally Pegg (1970) talked of "rapid" and "slow" cooling rates having been developed for both laboratory and transfusion service use. He seems at times to be describing techniques of slow and rapid cooling rates but at other times one gets the impression he is distinguishing a "rapid" technique as one where no post thaw washing away of cryoprotectant is required from a "slow" technique where post thaw washing is required.

Nor does the confusion end here since some authors refer to cooling rates without cryoprotectant additive being present, whereas others refer to their use. This point is clearly brought out by Mazur et al (1970). Thus

the optimal cooling rate for unprotected red cells was 2500°C to 3000°C/min (40 to 50°C/sec) whereas the addition of 20% glycerol lowered this to an optimum of below 1500°C/min (25°C/sec).

Regardless of the cooling and warming rate, most cells fail to survive freezing in the absence of a protective additive (Meryman, 1966). Cells which are cooled too rapidly are subject to intracellular ice formation which, although in very small crystals, can grow to damaging size or shape by the process of recrystallisation during thawing (Mazur, 1966; Luyet 1970). This suggests that one possible action of penetrative intracellular additives (e.g. glycerol, Dimethyl sulphoxide) is to prevent this recrystallisation on thawing.

Turner (1968) tried to reconcile these various confusions by defining as follows:-

(a) Ultrarapid freezing (a cooling rate of 100 to 1000°C/min in which the velocity is so rapid that the fluid cannot freeze by heterogenous nucleation and more numerous small ice crystals may form by homogenous nucleation throughout the material including inside and outside the cells. Provided thawing is equally rapid recovery of intact cells is favourable (Luyet and Menz 1950).

(b) Rapid freezing (a cooling rate at which intracellular ice crystals are produced which can do mechanical damage. It is necessary to prevent abnormal growth of damaging ice crystals which would cause mechanical damage, hyperosmicity and rapid denaturation.

Rinfret (1960) further extended this system by using glucose as additive and rapid freezing of bulk quantities

of blood with recoveries of 95 to 98% instead of 65 to 85% without additive. (Meryman 1965) To achieve such rapid cooling blood droplets were frozen by spraying a fine jet over the surface of liquid nitrogen (Meryman and Kafig 1955; Meryman 1965; Rowe and Allan 1965). Earlier work of rapidly freezing thin layers of blood in flat metal containers invented by Strumia (1962) established the fact that it would be possible to prepare cells and maintain them in a sterile condition with later satisfactory recovery (Strumia et al 1958, 1960) since the thin aluminium or tinned copper walls have minimal thermal resistance thus virtually reproducing the conditions of droplet freezing, (Florio et al 1943).

From earlier thermodynamic studies on heat transfer in containers Rinfret (1960) showed that container design was important. Crowley et al (1961) improved the design of containers and revealed that practically it is important to keep the layer of blood being frozen thin enough to allow rapid heat transfer. This could be improved by agitating the vessel as was introduced in the Linde (Hurn 1968) process and by the use of aluminium canisters with a corrugated profile to improve surface area. This is even more important during thawing since the principal resistance to heat transfer is the immediately thawed liquid layer inside the container. If this zone is vigorously shaken it will transmit heat by convection rather than conduction thus lowering its thermal resistance. Crowley and group also showed the importance of coating the outside of the containers with a thin layer of insulating material to avoid the formation of a high - resistance gas layer which

covers the outside of the container during the cooling in liquid nitrogen as a stable film of vapourised nitrogen. A variety of insulators have been used including vaseline, glycerol, polyvinyl pyrrolidone and paint lacquer (Doebbler et al 1966). The most convenient substance is the white thin lacquer coating modern cannisters (Jenkins and Blagdon 1971).

(c) Slow freezing is one in which ice crystals form only outside the cells eg. as in a domestic conventional deep freezer. Salt injury is most likely in this type since solute concentrates becomes hypertonic as water is used to make ice crystals. Glycerol is used to prevent this by its hygroscopic properties and this forms the basis of the High glycerol slow freeze technique of Huggins (1965). The principle obstacle to the widespread adoption of this system has been the necessity for expensive processing apparatus and the amount of washing post thaw to obtain glycerol free product for transfusion. A sequence of wash solutions of varying compositions and hypertonicity is required to minimise osmotic haemolysis. The excess hypertonicity of these solutions especially if they are non-electrolytes, can lead to electrolyte loss from the cells. (see p180)

Rowe (1968) considers moderate cooling rate to be 300°C/min and Krijnen et al (1964) defined "intermediate" cooling as 80°C to 120°C/min although Hurn (1968) called this "moderate".

It is to be hoped that these more rigid definitions

will answer the criticism made by Meryman (1968) that "the definition and measurement of freezing rate, a parameter basic to all cryobiology is quite without standardisation".

MODE OF ACTION OF CRYOPROTECTIVE

Lovelock (1952) gave a detailed and critical analysis of freezing injury in the human red cell. He concluded that freezing and thawing cause haemolysis because freezing subjects cells to extracellular concentrations of electrolyte (sodium chloride) above 0.8M, and because thawing subjects them to dilution. He showed that glycerol and dimethyl sulphoxide prevent haemolysis by preventing the electrolytes from concentrating to the critical value. The total mole fraction of solute in a partially frozen solution is determined by temperature. If all the solutes are electrolytes, then the required mole fraction will consist entirely of electrolytes, but if a non-electrolyte such as glycerol is present, the concentration of electrolyte will be reduced and the extent of reduction at a given temperature will be approximately proportional to the osmolar ratio of glycerol to electrolyte. He stated that only low molecular weight hydrophilic solutes with low eutectic points could protect cells, because only such solutes could yield solutions of high molar concentration at sufficiently low temperatures. To be protective a solute must permeate a cell, for otherwise it could not prevent a rise in extracellular electrolyte during freezing.

Despite these studies others such as Meryman (1968) and Leavitt (1966) believe that freeze injury is due more to removal of water and cell shrinkage (Meryman 1956, 1970).

Yet others believe that freezing causes a third effect namely damage by injury to the lipoprotein layer

of the cell membrane (Mazur 1970) and that additives protect the cell membrane from such injury. Hydrogen ion bonding of the protective solute acting as a stabilising influence on the hydration at the cell surface which if lost results in membrane damage. (Doebbler and Rinfret 1962) This was later also put forward by Valeri, Bond and McCallum (1966). Freezing may remove absorbed water from proteins leading to protein denaturation (Meryman 1970).

It is now clear that penetration of additives into the cell though desirable for maximum survival is not essential. More cells are protected with non-penetrating additives than without additives.

Perhaps all three play a partial role. There are few ways of avoiding ice crystal formation:- either freezing so rapidly that crystals do not have time to form - impossible with large volumes of blood, or less rapid cooling with additives of extra-cellular non-penetrating molecules to bind the extra-cellular water or even less rapid (slow) cooling rates and use of an endocelluiar penetrative additive such as glycerol or Dimethylsulphoxide.

The three hypotheses on the mechanism by which cells are damaged by freezing are: (Mazur 1968; Mazur et al 1970)

(a) The growth of ice crystals causes mechanical injury by crushing or shearing cells. Luyet & Gehenio (1940), however showed that the presence of extracellular ice probably does not exert any pressure on cells and certainly does not puncture them. Intracellular ice formation only occurs in cells in states of

of very rapid rates of cooling which occurs very rarely.

(b) Dehydration and concentration of the cell contents. Proteins may have an absorbed layer of water as a mono-layer which contributes to the stability of the protein itself (Sinanoğlu & Abdulnur 1965). Formation of ice may strip off this mono-layer leading to denaturation (Karow & Webb 1965). Levitt (1962) proposed that removal of water from the cell can lead to the distance between proteins or portions of proteins being reduced and that, as they get nearer to each other, abnormal disulphide bonds can be formed either through disulphide interchange or the oxidation of sulphydryl bonds.

(c) The concentration of electrolytes produced by freezing out of water leads to freezing haemolysis in red cells when the extracellular salt concentration changes from normal of 0.16M to 0.8M sodium chloride (Lovelock 1953).

In support of this Meryman (1959) showed that the mean corpuscular volume of erythrocytes suspended in steadily increasing concentration of sodium chloride in plasma with added sodium chloride showed the expected decrease in volume as the suspending solution was increased to 1100M - osmolal, or about four times isotonic. Above this the cells become leaky and succumb with solute (NaCl) passing into the cell and potassium passing out of the cell within 90 minutes. These leaky cells do not haemolyse but do so

readily on return to an isotonic solution where upon the grossly hypertonic cells undergo immediate osmotic lysis. Frozen cells would undergo similar changes during cooling leading to grossly hypertonic cells which would return to an isotonic medium on thawing with consequent osmotic haemolysis.

Meryman (1970) further extended this "salt lysis" theory to include his "minimum cell volume" theory which proposed that the development of extra-cellular ice leads to a concentration of those extra-cellular solutes which do not normally penetrate the cell. Water leaves the cell and osmotic equilibrum is maintained across the membrane. With continuing cell volume reduction and the compression of cell contents, a resistance to further shrinkage develops. If the cell cannot shrink freely in response to the concentration gradient, then an osmotic pressure difference must develop across the membrane. When this pressure gradient exceeds the tolerance of the membrane, irreversible changes in membrane permeability result.

There are two basic categories of cryoprotective agents:- those that penetrate the erythrocytes and those that remain extracellular. Some agents are used alone and others in combination with other additives. Included among the endocellular or intracellular additives are glycerol, dimethyl sulphoxide (DMSO), ethylene, diethylene, triethylene and propyleneglycols, acetamine, formamide; ethanol, methanol; monacetin, calcium lactobionate, and those compounds that penetrate the red cell membrane poorly such as glycerol monoacetate, erythritol, xylose and glucose.

The extracellular cryoprotective agents include dextrans and polyvinyl pyrollidone (PVP) of various weights, albumin, hydroxethyl starch (HES), hydroxypropyl starch, polyethylene oxide, polyethylene glycol, polyglycol (E50OM), multiatom alcohols such as mannitol and sorbitol, oxypolygelatins ('Haemacel', Gelifundol'), detergents and non-penetrating sugars such as lactose, maltose, sucrose and dextrose.

Investigations of the properties of various substances have been made since 1951. Since glycerol exerts its colligative effect principally by its non-specific effect of lowering the freezing point of aqueous media a number of other compounds have been found to exert a similar effect (Colligo-legi-lectum-to gather together into one place) These are mainly neutral diffusable substances of low molecular weight such as those on the list above. Doebbler and Rinfret (1962) tested 27 compounds for their cryoprotective qualities in preventing haemolysis of red cells on freezing. Included were alcohols, glycols and derivatives, sugars, amino acids, peptides

and the sodium salts of carboxylic acids. Lovelock (1954) tested compounds with several hydroxy groups including glycols, erythritol, and sugars. Methanol, ethanol, acetamide, formamide, ethylene and propylene glycols, diethylene and triethylene glycols, glycerol and monacetin were found to penetrate the erythrocytes well. Poorly penetrating solutes included erythritol, xylose, and glucose. Non-penetrating solutes included sucrose and polyethylene glycol.

Vinogard-Finkel (1959) used various combinations of similar preparations. Sloviter(Abst. 1954) used polyhydroxy compounds. Doebbler and Rinfret (1962) noted that not all additives were capable of penetrating the cells and protecting to the same degree or with the same efficiency on a molar basis. Nash (1962) analysed the chemical similarities of these neutral penetrative solutes and concluded that the following can give complete protection against lysis by freezing to any temperature down to -79°C in 15 minutes:-

dimethyl sulphoxide, dimethyl acetamide, dimethyl formamide, methyl acetamide, ethylene glycol and glycerol. Pyridine - N - oxide gave complete protection down to -40°C.

Doebbler and Rinfret (1965) showed that the percentage protection for 1 Molar concentrations were glycerol 94%, monoacetate 41%, glycerol diacetate 2%. For glucose and derivatives the protective activity was: 0.5M glucose 88%; 0.5M alpha methyl glucose 88%; 0.25M alpha methyl glucose tetraacetate 48%; 0.5M sodium glucuronate 28%.

Chanutin and Curnish (1960) found that the protective effect of glycerol, DMSO, glucose and sucrose against methaemoglobin formation by freezing might be due to their altering the structure of the ice crystals surrounding the haemoglobin molecule.

Grieff and Seifert (1968) produced some evidence in chicken red cell work to support the suggestion of Ho et al (1962) that these protectives might in some way bind to cryosensitive sites on the cell membrane.

Turning to the choice of non-penetrating protectants, Pert et al (1963 Abst) studied the cryo-protective qualities of glucose-lactose, albumin, PVP, DMSO, dextran, glycerol and glycerol-sucrose. They found that the most satisfactory results were obtained using 14% (w/v) glycerol, and 2.76% (w/v) sucrose with static freezing at $-120^{\circ}C$ with storage at $-170^{\circ}C$. Luyet (1965) summarised the principal cryoprotective substances which had been studied up till that time.

From this brief look at some of the more interesting aspects of cryoprotectant solutions it seems clear that we should concentrate on these two aspects (a) intracellular additives and (b) extracellular additives.

(a) Intracellular additives

It has already been noted that a chance observation led Polge (1949) to investigate the action of glycerol and related compounds in protecting spermatozoa and contaminating red cells against the effect of low temperatures. Thereafter Smith (1950) reported the action of glycerol in preventing

haemolysis due to freezing and thawing. Parks (1951) reported freezing of guinea pig and rabbit blood protected with 10–15% glycerol (w/v) and storage for three months at -79°C. Sloviter (1951, 1951a) used glycerol to prevent haemolysis, of rabbit and human erythrocytes. Tullis (1965) discussed how glycerol could replace water in the cell. When the amount of water is below 20–30%, the remaining liquid is so tightly bound to cellular protein that freezing will not occur at any temperature or rate of heat exchange. If it is assumed that the normal water content of a red cell is 72 gm/100 cc and the whole blood is 83 gm/100 cc it would require from 40 to 50% glycerol to effectively displace the aqueous phase down to a concentration of 20–30% and thus make glycerolised red cells independent of rates of freezing and thawing. This is actually the concentration of glycerol recommended by Huggins and Tullis for optimal preservation of red cells in the slow freeze (mechanical refrigeration) method using storage at -80°C previously reported by Tullis in 1962.

Such heavily glycerolised cells required some form of deglycerolisation before infusion since if infused in a "loaded" condition, water is rapidly sucked into the glycerolised cells which then distend and undergo osmotic lysis. Such deglycerolisation was first tried by semipermeable membrane dialysis (Sloviter, 1951) but because of the slowness and lack of sterility this method is of no therapeutic value. Centrifugal removal of supernatant glycerol is easily achieved (Chaplin & Veall 1953) and more recently this has been coupled with stepwise addition of osmotically balancing solutes to

prevent cell rupture either by wringing them dry using hypertonic salt solutions or addition of sodium citrate to 0.33M or glucose to 1.0M (Lovelock 1954).

In 1965 Huggins and Grove-Rasmussen reported that glycerol in concentrations of 4.8 to 5.6 Molar enabled red cells to be stored at temperatures below minus 80^{o}C with a minimal necessity for controlling the rate of freezing and thawing. Pyle (1964) further stated that at glycerol concentrations below 16% the velocity of freezing and thawing and the storage temperature became increasingly important.

Carlsson et al (1967) decided that for military stock-piling glycerol should be the most appropriate protective substance because it caused only minor cell loss and yielded a product satisfactory for transfusion. Krijnen et al (1965) used a low glycerol rapid freeze technique with 4% sorbitol added to the glycerol and saline solution. A final concentration of 17.5% (w/v) glycerol was found to be better than lower concentrations used. In 1970 the author visited Amsterdam to see this system in use and wrote a detailed report to the Scottish National Blood Transfusion Association (see appendix). A visit at this time was also made to the Army Blood Supply Depot at Aldershot where similar methods of preservation were being used. As a result of these visits an application was made to S.N.B.T.A. for a development grant of £3000 to study the feasibility of setting up a bank of frozen cells in the Western Region of Scotland and which formed the basis of the present study (see p 68).

Pert et al (1967) combined a formulation of 16% glycerol (w/v) with sucrose (2.8 gm/100 ml) to protect erythrocytes during rapid freezing and thawing, storage being maintained at -120°C. Rapatz and Luyet (1967) studied the problems of optimal cooling rates with varying concentrations of glycerol. Generally they found that with slower rates of cooling there was a progressive increase in protection with increased concentration of protectant. They concluded that the addition of high concentration of glycerol inhibited or reduced the formation of damaging intracellular ice. They also showed a similar effect with varying concentrations of the other penetrative additive DMSO which was found better for preservation of nucleated erythrocytes of salmonidae by Hodgins and Ridgway (1964). Huggins (1963) reported the successful use of DMSO in protecting large volumes of human erythrocytes against slow freeze thaw haemolysis. Huggins (1964) further described in detail a technique for freezing and preparing such cells for use. Richards et al (1964) however commented on the unpleasant smell of dimethylsulphide acquired by patients transfused with DMSO preserved cells. DMSO has been shown to cause lens opacity in dogs in high dosage. The material is exothermic and requires careful dilution prior to use. Hurn reported that Huggins by 1965 had abandoned the use of DMSO although it is still used today in ox red cell freezing programmes (Dobry et al 1968).

(b) Extracellular additives

Strumia, Caldwell and Strumia (1958) first indicated that it was possible to freeze and thaw blood in bulk

(25-50 ml) containers designed to permit rapid heat transfer and to recover 95% of the red cells. The blood was modified with lactose or glucose alone and in combination gave reasonable in vivo survival although the cells were functionally poor. Cells were not washed before infusion and only some supernatant was removed to partially pack the contents and remove free haemoglobin. Since it was argued that these sugars were naturally occurring substances they should be ideal if they could be made to give cryoprotection but unfortunately Rinfret et al (1965) reported that the amounts of lactose required to give good cryoprotection was lethal to dogs and therefore a wash stage would be required although Strumia and Strumia (1963) had transfused 15-20 gram lactose in 500 ml blood without difficulty in healthy individuals. They recommended the use of polyvinylpyrrolidon and dextran with albumin as an addition to lower the concentration of P.V.P. Rapid freezing and thawing was required in these methods and Rinfret (1963) working with the Linde Company described their Linde processor system consisting of a corrugated metal container which allowed a 4 mm thickness of modified blood to freeze over a large surface area during rapid agitation in a bath of liquid nitrogen. In further experiments Rinfret et al (1964, 1965), Pert et al (1965) were able to define the conditions more closely. Highest recovery and stability of recovered cells was obtained with PVP/albumin mixtures and confirmed the work of Bricka and Bessis (1955) that P.V.P. would provide a high degree of protection (see also Glauser and Talbot 1956, Steinbuch and Quentin 1958, Doebbler and Rinfret 1962).

Furthermore the macromolecular substances do not penetrate the cells and so recovered cells from the frozen bank are not hyperosmotic and do not undergo rapid osmotic lysis when transferred to an isotonic environment and, provided they could be shown to be non-toxic to humans, they need not be removed before transfusion. Clearly the ideal would be indefinite storage, easy transport, minimum manipulation, additive safety, maximum recovery and red cell survival. Despite the toxicity of lactose already referred to, it was expected that if a satisfactory method of storage and recovery could be achieved then the toxicity of the preservative could be removed by centrifugation and decantation prior to administration. For this and other reasons Strumia and Strumia (1965) persisted with the development of Teflon bags frozen between fluted (corrugated) copper plates to produce a thin film of blood and preservative for freezing (0.4 cm thick) spread out over a large surface area all the while vigorously agitating the bag. Although good recoveries were obtained (greater than 97% in all cases) the mean red cell survival at 24 hours in the circulation was 66.9 ± 6.3 (T/2=22 days) for dextran 40 protected cells and 62.6% (T/2 = 31 days) for albumin protected cells. Unacceptably high levels of post recovery free haemoglobin and supernatant potassium were common and again indicated the need for a centrifugation step to ensure transference of these substances prior to transfusion into a recipient. Since they did not offer anything more than other techniques and indeed the final decantation step denied the very first tenet of the

system - that no washing or other manoeuvre be needed - the system has not found favour.

Furthermore the original work of Strumia and Strumia (1965) showed that oxygen dissociation curves showed a distinct shift to the left, i.e. blood originally 1 day old when frozen and thawed behaved like blood 20 days old in ACD. This defeated another tenet of the system - that the final product should be physiologically equal to or superior than conventional ACD liquid stored blood.

Further extensions of the work in P.V.P. were reported by Vinograd-Finkel et al (1971) following work done with the Linde Companies' system by Gikas et al (1965). Using the standard corrugated aluminium can and liquid nitrogen, 4 day old blood was preserved in 7.5% P.V.P. with recoveries reported as high as 96.5% and post transfusion survival of at least 70%. In the monkey average recovery was 95.3% (82.5-98.0%). No differences were found with P.V.P. of molecular weights 25,000 or 40,000. Post thaw potassium levels were 18.4 meq/litre and similar to that found in conventionally banked blood 14 days old. (Monkey plasma potassium is the same level as man). Plasma haemoglobin was 284 mgm/100 ml (range 87-850 mgm/100 ml).

Despite these somewhat poor recoveries some workers still persist in their efforts to achieve what is the ultimate goal of all blood freezers: to obtain an additive which will protect the cells against freeze injury and which itself is not harmful to a recipient, does not interfere with normal body function, results in a first-class recovery in the minimum of time, does not require any subsequent manipulation or further processing and is

not stored in the reticulo-endothelial tissues (Ravin et al 1952) and will preferably be rapidly and completely metabolised in the body. These desirable features are attractive to blood transfusionists with a special interest in long term preservation and stock piling of blood to meet unexpected and heavy demand in mass casualty situations both in civilian and military practice. (Strumia and Strumia 1965; Crosby 1967; Vinograd-Finkel 1971 a and b; Robson 1970). Robson in reviewing his work with PVP of various molecular weights believed that the problems of long term storage in the reticulo-endothelial system described by Ravin et al (1952) need not be troublesome provided low molecular weight varieties were used. Using 7.5% concentration in ACD blood donations, 96.4 $\pm$ 0.5% recovery could be achieved using the aluminium corrugated can system with liquid nitrogen in the Linde processor. Saline stability was 84-86% and in vivo survival averaged 73.5% at 24 hours post transfusion which remained fairly uniform post thaw storage at 4^{o}C up to 12 days after which they rapidly deteriorated. Again unfortunately one is forced to conclude that the overall efficiency is much below that of glycerol methods of freeze preservation and high levels of supernatant haemoglobin and products of cell death are to be expected unless washed away. It is not therefore just a question of loss of some cells which, if the product lacks quality, can be made up by quantity and provision of additional frozen - thawed material. (MacFarlane 1964).

Because all of the detritus remains there is to be

expected results similar to those reported by Gikas et al (1965) and Thompson et al (1965):- In monkeys under conditions of stress, such as exsanguination and haemorrhagic shock, where although the cells were capable of sustaining life there was evidence of marked haemoglobinuria in all cases and renal microtubular injury in some which was reversible and did not interfere with renal function. Nonetheless these experiences must make us cautious in the use of P.V.P. stored red cells in civilian practice where there is usually adequate time to obtain more physiologically superior cells from a low glycerol - rapid freeze bank either in response to a request or as a planned procedure.

In 1967 Knorpp investigated the cryoprotective properties of a recently discovered volume expander hydroxyethyl starch which had the attractive property that, being a starch like substance, it could be hydrolysed by body amylases and so when administered would not accumulate but be metabolised. This surely was another example of the search for the ideal preservative and seemed to answer one of the desired features - that it should be non-toxic and not stored in tissues. As a 15% concentration he used it to protect 55 ml aliquots of whole human blood against injury during rapid freezing in liquid nitrogen and rapid thawing in a water bath. 97.4% recovery was achieved in vitro (as good as 7% P.V.P. reported by Knorpp et al 1966). Robson (1970) reported a determined attempt to scale up the process to preserve whole donations of blood begun by repeating similar experiments in small aluminium containers. Recoveries of

96.5-97% were obtainable with post thaw saline stabilities of 86-90%. When HES was added to whole donations there was a dilutional fall in haematocrit and the final fluid volume was 800 ml - too much for the available freezing methods. Further attempts using anticoagulant dissolved in 40% solutions of HES although resulting in satisfactory haematocrits and a post thaw recovery of 96.8% gave only 80.5% saline stability. Suspending packed red blood cells in HES directly resulted in even worse figures of 96.8% recovery but only 80.5% saline stability. Finally the 40% HES was added in a volume equivalent to the volume of plasma removed from red cells to give a final concentration of 15% with a haematocrit of 0.37 and recovery of 96.9% of which 89.2% was stable in saline after 30 minutes. Haemoglobins in the supernatant were recorded as being sometimes in excess of 2 gm/unit of frozen blood and caution was again urged before this method could be used clinically.

Dobry and Livera (1962) used dextran to preserve cells against freezing to -70°C and Dobbler and Rinfret (1962) obtained 90% recovery of 7% dextran protected

cells, (7% polyethylene glycol protection gave recoveries of 91% and 95% respectively). Similarly Strumia (1962) reported that albumin 17-22 gm/100 ml and dextran 15-21 gm/100 ml (molecular weight 40,000), would protect 93.5% of cells from damage. Post transfusion survival was poor (40% loss) however - a fact previously noted by Doebbler and Rinfret (1962) and Strumia and Strumia (1962). Although the dextran yielded a good recovery of cells immediately after thawing their stability in isotonic saline was greatly reduced. This post thaw saline instability of cells recovered by a variety of methods had earlier been seen by Doebbler and Rinfret (1959). In a series of reports Strumia (1964, 1965) studied the additive effect of additional lactose with human albumin and dextran concluding that prior treatment of red cells with lactose enhanced the survival of dextran or albumin treated cells. Bloom (1965) reported that the protective action of PVP was the most satisfactory than any other additive. Hurn (1968) stated that in the Linde (liquid nitrogen) method a mixture of PVP and albumin (final concentrations 7% and 1.5% v/v respectively) gave satisfactory results. This combination of PVP/ albumin had been previously noted by Rinfret (1963). Such polymers give in vivo survivals after recovery as good as conventionally banked blood. Steinbuch and Quentin (1968) used PVP/plasma albumin also for cryoprotection. Doebbler et al (1966) concluded that PVP was superior to other polymers, including dextrans, gelatins and oxypolygelatin with an optimum molecular size of 17K PVP at 20,000.

In 1967 Knorpp et al investigated the use of the recently discovered volume expander hydroxyethyl starch. (HES) This has the advantage that it is hydrolysed by body amylases to glucose and thus prolonged retention in the tissues is avoided. It is as effective as PVP at a final concentrations of 15% (v/v) and may be used as a one-stage recovery procedure. Hydroxypropyl starch has similarly been tried (Knorpp et al 1968) but has no better advantage than HES. (Brickman et al 1966).

Other saccharides have of course been used frequently in the past. Thus the earliest recorded freeze preservations and red cells used glucose 0.5 ml of a 20% solution to 1 ml of blood in aluminium containers and freezing at 20°C/ml. 97-99% recovered were claimed (Florio 1943). Meryman and Kafig (1955) showed that 7% glucose gave 50-85% recovery of red cells frozen as droplets in liquid nitrogen. These results have all been confirmed by Strumia et al (1958, 1960), Huntsman et al (1960), Doebbler and Rinfret (1962), Rinfret (1963), Hurn (1964). Many other sugars of a non-cell penetrating type have nevertheless protective properties. All sugars whether penetrating intracellular protectant or non-penetrating extra-cellular protectant have the disadvantage that they can cause a profound effect on plasma osmotic pressure of the recipient, since being small molecules they exert a considerable effect on osmosis and result in sudden increases in blood volume. Since the goal is a product which can be transfused safely without any post - thaw processing these findings of Pert et al (1962) led those interested in

the therapeutic use of frozen blood to abandon sugar for other types of protectant. Nevertheless the commonly used glycerol protectives must also be removed from the cells before infusion to prevent osmotic lysis in vivo.

Rinfret (1963) used a 10% solution of "Haemacel", a cross linked partially degraded gelatin, as a freeze - thaw protective blood additive. Unlike other polymers it is synthesised from protein and can be metabolised (Moebler and Sykudes 1962). Unfortunately its protective effect is no greater than albumin and varies from 60% to 80% when rinsed with sucrose or sucrose sodium chloride.

Summary

It seems from the foregoing account of cryo-protectives that simple sugars and disaccharides are of limited application for the storage of panels of cells rather than for transfusion purposes because of (a) their osmotic effect on the recipients plasma and (b) the water effect on cell size with penetrative endocellular molecules; polymers of human and non-human origin, albumin and polygelatins give poor recoveries as do polysaccharides of the dextran hydroxyethyl and propylethyl starch variety. Non animate polymers such as polyvinyl pyrollidone may be stored in tissues especially the high molecular weight forms. Doubts about toxicity in man have not been resolved although in vitro PVP is probably the most satisfactory single substance for freeze protection giving greater than 90% recovery of red cells. We are therefore left with the first substance to be found and studied - glycerol, now known to be not only

cryoprotective in vitro but also established as the likely substance providing the essential clue to survival of plants and animals at sub-zero temperatures on land and in the polar seas. It seems incredible that after all the work and searching for the ideal preservative protectant that the original accidental discovery of Polge (1949) is still the most valid having stood the test of time.

Glycerol is of course acquired in normal body fat metabolism (Triglyceride formation) and without any effect on red cell metabolism (Tullis and Lionetti 1966). It can be given in high concentration intravenously without adverse effect. Sloviter and Ravdin (1958) gave 50 gram intravenously without adverse effect. Indeed Meyer et al (1971) used a 10% w/v solution in neuro-surgery for osmotically shrinking brain tissue. Its effect on red cells is similarly purely an osmotic one related to its concentration. Nevertheless it is important to remove it from within the freeze preserved red cells so as to avoid the rapid ingress of water whenever the cells are recovered and returned to an isotonic medium (or plasma of transfused recipient). This is best achieved by stepwise removal of glycerol by addition of hypertonic osmotically active substances (sorbitol, mannitol, sucrose) to minimize the shock to the red cells and allow the process of glycerol removal to occur fairly slowly and at a measured rate by osmotically "wringing" the red cells free of glycerol ("osmotic squeezing"). This process has already been discussed and is the basis of the modern deglycerolization methods used in batch washing and which are described in this text.

STORAGE OF RED BLOOD CELLS

The mammalian red cell is in many respects unique among cells: it possesses neither a nucleus nor any other detectable internal structure; its shape is a biconcave disc; its outer membrane is very freely permeable to water but is relatively rigid so that the cell is more restricted than in nucleated cells; and there is no means of replenishing effete protein molecules. These features are reflected in unique conditions required for optimal preservation.

Stored erythrocytes are used for two main purposes: small quantities of the order of 1 ml are used in serological tests. Large quantities of the order of 500 ml are used for blood transfusion. The practical value of banks of frozen cells can hardly be exaggerated. The availability of typed reference cells for blood grouping and the identification of red cell antibodies has contributed significantly to the present day safety of blood transfusion. Similarly in detecting unusual and rare antibodies there is a need to store cells for transfusion to such patients if the need arises especially in emergency and semi-emergency situations where there is insufficient time to select suitable donors. In addition autologous blood storage in such cases becomes possible. Military medicine has strategic reasons for the preparation of banks of frozen cells where under battle or mass casualty conditions stock piles of blood can be released at short notice. Numerous studies have appeared in recent years indicating that frozen cells offer advantages over conventionally banked blood in that unwanted and potentially

harmful cations, leucocytes, platelets and plasma components can be removed including micro emboli, hepatitis (HBsAg) and other viruses. Each of these will be reviewed in detail. More recently we have specifically used frozen cells for the induction and boosting of human volunteers for the production of human anti-D immunoglobulin and there seems no reason that such studies could not be extended to other red cell antigens and antibodies.

Freezing of Small Samples

The rapid freezing of small samples of erythrocytes is relatively easy and numerous methods have been described (Crawford et al 1954). Sterility is not a pre-requisite and the methods are therefore of the open non sterile type. Meryman and Kafig (1955) described a method of spraying blood from a fine (0.18 mm) syringe needle onto the surface of liquid nitrogen. Droplet freezing rapidly occurs and separately droplets (0.45-0.9 mm diameter) can be harvested from the freezing vessel, when they can be stored for later recovery in 42°C saline suspension by simple thawing addition (Meryman 1956). Addition of 0.4 Molar glucose provides better cell survival from 50% up to 95%. Huntsman et al (1960, 1962, 1964) found 0.4 Molar sucrose even better and recommended dilution of fresh blood with half its volume of 1.2 M sucrose immediately before dropwise addition into liquid nitrogen. Storage temperature of the droplets ("blood sand") showed that at -60°C deterioration occurred at 2% per week at -130°C; at lower than this the storage time was indefinite. Such a temperature is easily maintained in the vapour phase of a liquid nitrogen filled container (-150°C). Since Meryman's description numerous workers have reported variations in procedure. (Burnie 1965; Gibbs et al 1962; Huntsman et al 1960; Krijnen et al 1964; Mohn et al 1970 and Rowe and Allan 1965). Comparisons of viability of fresh and stored erythrocytes when used for antibody identification have been made and found to be satisfactory (Bronson et al 1962; Huntsman et al 1960; 1963, 1964 and Seidl, 1972; Strumia 1962). As well as these earlier methods it is now well established that liquid nitrogen provides the most

effective method for red blood cell freezing (Rowe 1970, 1971; De. Verdier et al 1965; Baar 1973, Bowman et al 1973, Rowe et al 1967; Akerblom and Hogman 1970, 1974; Reid and Ellison 1974).

Freezing in Bulk

Normally workers were interested in storing whole donations of blood but early attempts trying to scale up these small bulk storage methods met insuperable problems over sterility. Strumia et al (1960) however did succeed where others had failed by using blood sealed in flat metal containers with sugar additives such as glucose or lactose. Thereafter the Linde division of Union Carbide Corporation developed an automatic blood processing machine in which pint volumes of whole blood could be cooled and thawed rapidly. By the use of thin corrugated containers with mechanical agitation in a bath of liquid nitrogen the cooling rate was sufficiently rapid for good cell survival. Unfortunately the concentration of sugar additives resulted either in rapid destruction of a large proportion of the cells during the first few minutes following transfusion or else the additives themselves produced unacceptable pharmacological actions in the recipient. Other additives were tried including polyvinylpyrrolidone (P.V.P.), dextrans, glycerol and more recently hydroxyethyl starch and oxypolygelatins. In most of these however the additive must be removed after thawing which increases the recovery time and the risk of bacterial contamination.

The expectations for a frozen cell programme include indefinite storage, simplicity of processing, utility and availability of the product, clinical acceptability and low costs. The ideal would be red cell preservation by freezing with production of the same cells which after thawing require no additional processing. The particular method chosen depends on the need to remove or leave the additives and products of haemolysis before transfusion. No "one stage" process has yielded biologically acceptable cells and at least some post thaw processing has been necessary either by washing the thawed red cells with or without removal of the supernatant fluid by either centrifugation or sedimentation (the two step method). Valeri (1966) shows the variety of methods available for Red Cell Preservation by freezing up to that date.

Methods of Choice available using Glycerol as Cryoprotectant

A. The High Concentration Glycerol Technique (45% w/v) was not unexpectedly the first to be used since the work of Smith (1950) and Mollison et al (1952) appeared soon after the discovery of the cryoprotectant effect of polyalcohols at 80°C. More recently Pert et al (1964 and 1965) described the equally protective effect of glycerol in lower concentration (18% w/v) provided freezing could be more rapid using liquid nitrogen at -196°C. These studies were clinically evaluated by Krijnen et al 1964; Akerblom et al 1968 and Rowe et al 1968. Post thaw washing is essential in both of these methods in order to avoid haemolysis which would occur if heavily glycerolised cells come in contact with isotonic solution or blood

plasma (see p.51). The freezing techniques require special equipment for (1) the glycerolization prior to freezing. (2) the storage phase whether at -80°C in mechanical refrigerators or in the gas phase of liquid nitrogen (-150°C). (3) the thaw phase, and (4) the deglycerolization phase prior to transfusion. Each of these will be taken in turn and the advantages and disadvantages of each explored to justify the final choice of method.

1. The glycerolization phase. Packed cells should be allowed to warm up to room temperature since the glycerol flux is temperature dependent (Meryman and Hornblower 1972). In general, glycerolization in all methods should be done with rapid mixing since it has been well established that concentrated cells being exposed to high concentrations of glycerol can undergo hypertonic lysis. Most investigators add the glycerol whilst the cells are being mechanically shaken. We use a bench "Rotamix" model during the addition of cryoprotectant to the packed cells (see Technical appendix - method). Meryman and Hornblower (1971), also commented that unless the first 10-20 ml of glycerol were mixed rapidly with the cells there was a tendency for the glycerol to adhere as a film to the PVC plastic bag inner surface from where it is only slowly dispersed into the bag contents. Cells trapped in this static layer are subjected to excessive hypertonic osmotic stress. They therefore recommended the use of a plastic needle projecting 5 cm into the bags through which glycerol was added at a rate not in excess of 35 ml/min until 100 ml had been added

during continuous shaking at 4 cycles/second. Thereafter after a period of 5 minutes equilibration the additional 300 ml 0.2M or glycerol could be added before transfer to a PVC freezing pack for cooling to -80°C. With this careful attention to detail they were able to reduce the supernatant haemoglobin in the glycerolized cell suspensions pre-freezing from 50 to 150 mgm/dl to less than 20 mgm/dl, (475 units average 9.9 mgm/dl ± 8.6 pre-freeze and immediately post thawing to 9.6 mgm/dl ± 27).

2. The Storage Phase - Containers for Freezing. (Plate 1/4).

Nowadays containers are either metal usually high quality aluminium or some form of plastic. For the high glycerol methods cans are usually flat stainless steel (Meryman and Hornblower 1972; Krijnen et al 1963) or flat or corrugated aluminium to increase the available surface area for freezing (Doebbler et al 1966; Rowe et al 1969; Valeri 1971) Occasionally aluminium foil has been used held in a rigid restraining plate which gives the same flat appearance (Vinograd-Finkel et al 1962; 1969; 1971; Akerblom et al 1968, 1970; de Verdier et al 1965). Polyvinyl chloride (PVC) bags can be used for high glycerol -80°C storage and modified polyethylene (Teflon) can be used to withstand lower temperatures down to -196°C (liquid nitrogen) with named brands being Cryovac (Fenwal Laboratories) Hemoflex and Ucar (Union Carbide), Marlex (Pharmachem Corp.) Habia Kapton F.E.P. (Habia Corp). (Rowe et al 1968, 1971; Akerblom 1970; and

Plate 1/4. Containers for Freezing Blood.

Hornblower 1972).

The thickness of containers in low glycerol - rapid freeze methods is obviously more important than for high glycerol methods. In Pert's (1962) original method Teflon laminate bags were only 3 mm thick when filled, whereas in the Rowe method (1968) the stainless steel container was 6 mm thick and close to the upper limit found by Pert et al 1965. To increase the surface area in contact with the blood so as to give rapid even freezing and thawing, Doebbler et al (1966) described the use of corrugated aluminium cans of 0.5–0.8 mm thickness and rectangular cross section. Cans to take 30–75 ml were 7.6 x 7.6 x 1.9 cm and larger cans to take half-pint and pint volumes were 17.5 x 17.5 x 1.9 and 25 x 24 x 1.9 cm respectively. During freezing and thawing blood cans were rapidly aggitated. (Bloom et al 1960).

Akerblom (1970) using the flat sectioned Habia bag made of Teflon - FEP laminate showed that freezing thicknesses of 10–11 mm were obtained with 300 ml blood, 15 mm with 1100 ml blood and 20 mm with 500 ml blood. The system behaved quite differently from that with other flat containers. When immersed in liquid nitrogen directly, although the plastic withstood the temperature, the contained blood showed a 28% haemolysis compared to 2% from aluminium containers. If the bags were covered in a thin envelope of perforated aluminium sheet (0.05–0.2 mm) and provided the aluminium envelope was pressed tightly shut to squeeze the bag into a uniform thickness of blood, freezing was

satisfactory and cells could be recovered of the quality required. This technique of modifying the freezing rates using aluminium foil or plastic layers had been previously documented by Pert et al (1963, 1969). These latter authors also experimented with a variety of other designs of container including (a) circular aluminium cans designed so that freezing, thawing and washing in a centrifuge could be carried out without transfer of the blood to any other container. (b) circular Teflon bags designed so that freezing, thawing and washing in a centrifuge can be carried out without transfer of the blood. (c) rectangular metal cans for freezing and thawing with subsequent transfer to a circular centrifuge bowl for washing. (d) rectangular Teflon containers such that freezing, thawing and washing by agglomeration techniques can be carried out without transfer of the blood.

It can be seen therefore that this early work by Pert laid the whole basis for the different circular and rectangular cans and bags used for freezing today. They also established that 14-20% glycerol gave optimal protection in liquid nitrogen. This laid the foundation for the metal can procedure of Rowe (1967) and Krijnen (1969) which in turn was adapted by Jenkins and Blagdon (1971) and later by Pepper. Plastic laminates were to follow from Rowe (1968) and Akerblom (1969).
Pert who worked out the optimal freezing rates, correct geometry of the pack to be frozen and best thawing technique does not always receive the credit he deserves. One criticism of his techniques (1968) is the rather

cumbersome "cantilever freezing unit" designed to transfer the bag into the liquid nitrogen bath with a tilting device reminiscent of a mechanised transfer basket seen in some fish and chip shops!

Krijnen and co-workers (1971) reported an advance in the design of freezing containers instead of the expensive stainless steel flat containers in previous use. It consisted of an expanded (one piece) aluminium can with a specially designed gas-tight neck seal to prevent nitrogen leak which had proved troublesome in early experiments by causing explosive rupture at thawing. Coupled with this can the authors had developed a multitailed wash bag which allowed fluids to be passed in and out for washing by conventional refrigerated centrifuge. In their system the cooling rate is an average 0.7^{o}/sec and this rate can be expected to give a good recovery in the presence of 17.5% w/v glycerol (Pert and Schork 1968).

Akerblom and Hegman (1970, 1974) described the development and subsequent use of a cryostable plastic bag made of Teflon laminate (Habia bag) in which full donations could be nitrogen frozen and recovered with the cells retaining their metabolic integrity such that the ATP, 2,3 DPG and post transfusion survival figures were as good as for fresh ACD bank blood. Blood was frozen in a layer 12-13 mm thick between aluminium net and aluminium plate by static immersion in liquid nitrogen (5 minutes). Bags were then stored in 250 litre storage dewar vessels in rhomboidal aluminium baskets with each dewar holding 120 units of blood in a single

layer sitting in liquid nitrogen with the bag necks in the vapour phase. Thawing was by gentle agitation in a water bath at 37–40°C and took 3–4 minutes. Post thaw washing by serial centrifugation (batch washing) was done by using hypertonic sodium chloride solution (3.7%) followed by 3 washes with normal saline. Instead of glass bottles they recommended a plastic wash bag for the washing phase consisting of a 600 ml capacity bag with five tails of tubing for transfers. This bag is very similar to that produced by Tuta U.K. Limited early on in our phase 1 development which was developed as a single wash bag (multitail) following discussions between Drs. Jenkins and Blagdon, Brentwood and ourselves at B.T.S. Law. (see appendix). This in turn was a development of the Krijnen multitail wash bag system which had design features unsuitable for the U.K. users namely (1) metal needle piercing connections to the tails of the transfer lines and (2) a central giving port placed in the lower third of a full face (lateral wall) of the bag which required a metal piercing unit type giving set which in our view could lead to pin hole leaks of the opposite wall if the bag folded during use (Plates 1/5 and 1/6).

These authors were able to adopt this method so that a variety of recovery techniques could be used depending on which facilities were locally available. Thus it was possible to use batch washing and cytoagglomeration by two methods to achieve adequate cell recoveries and removal of excessive supernatant and cellular deglycerolisation. During thawing leaky

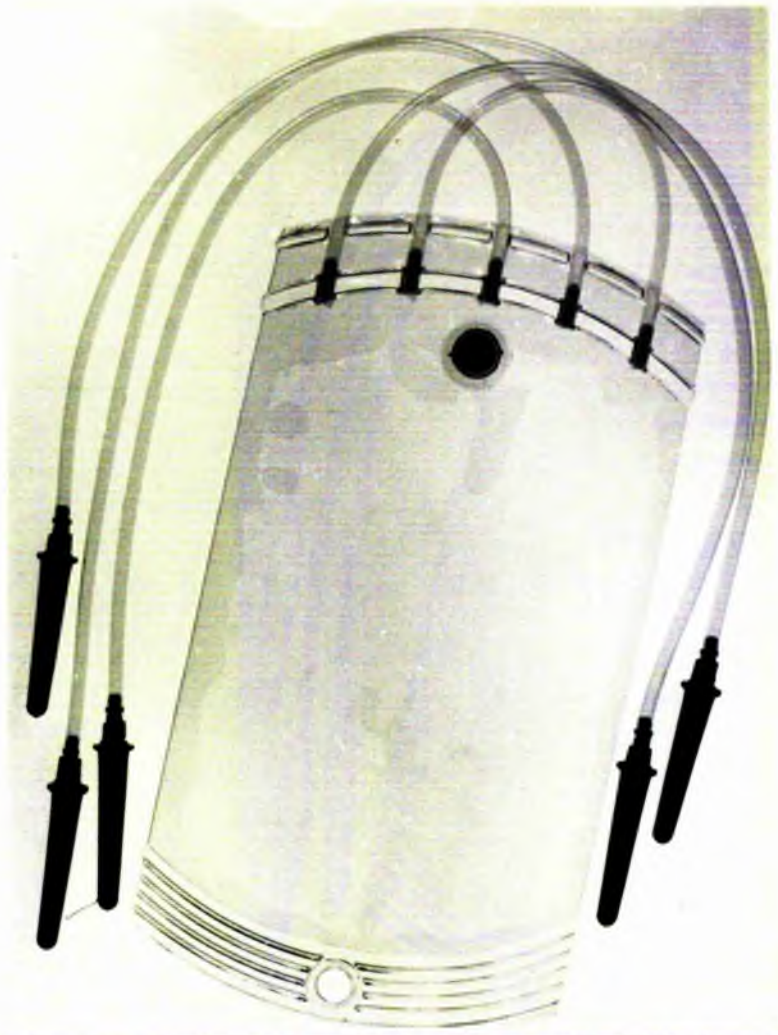


Plate 1/5
Amsterdam Bag

Plate I/6
Tuta(UK)
Bag

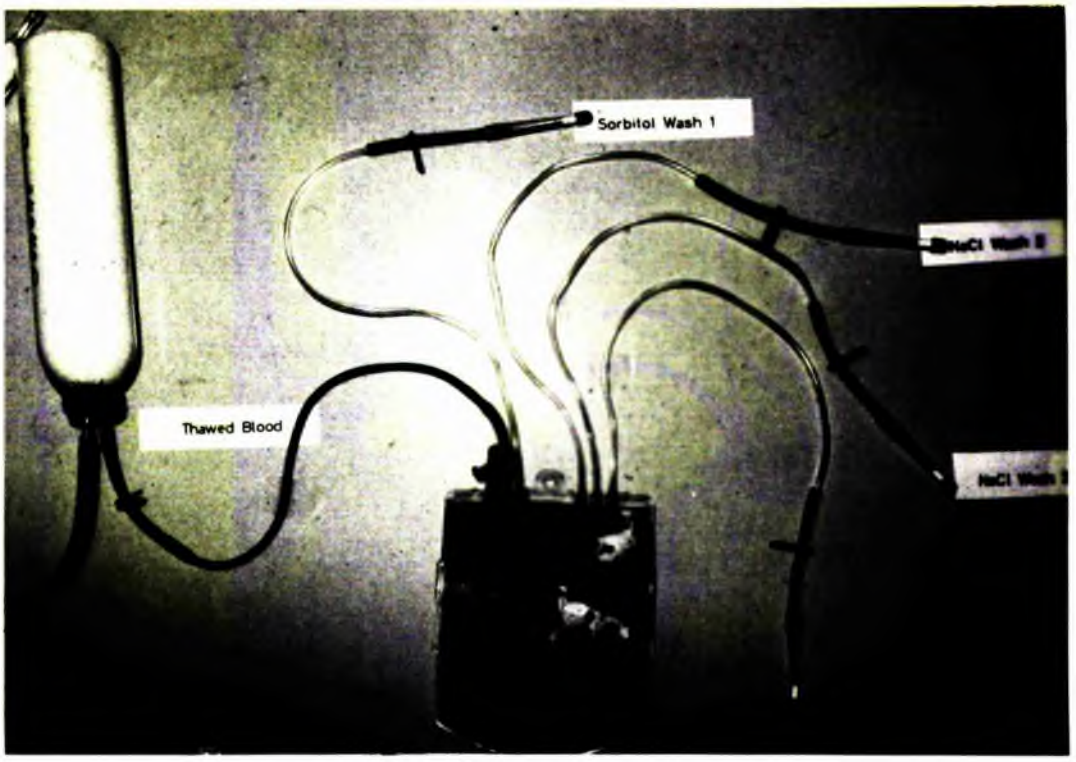


Plate 1/5 and 1/6 Multitail Recovery/Delivery Bags

bags due to salt from losses due to liquid nitrogen rupture similar to that described for metal containers.

This technique of low glycerol rapid freeze has important advantages over other methods - a minimum of special equipment is required (liquid nitrogen refrigeration, metal freezing containers or plastic cryo stable bags). Pre-freeze and post thaw processing can be performed using standard blood bank equipment. The metal container is however subject to serious explosions if liquid nitrogen accidently leaks into it. Labels might fall off in storage and pilot samples could not easily be stored with the frozen blood unit. In our system these problems have not proved to be insurmountable and modifications to design new developments have overcome these problems. (see p 90). They claimed that the Teflon laminate was resistant to mechanical strain. This has not been our experience and approximately 10% of sample bags burst at the welded seams during centrifugation. It is also claimed that the bag ports fit standard blood transfer sets but this was not our experience especially with early prototypes for trial testing in U.K. These same authors (1970) used these same bags to store smaller donations taken from dialysis patients for later auto transfusion. It was claimed that this method allows various forms of red cell recovery, manual, automated and agglomerated.

3. The Thaw Phase.

In general thawing is accomplished rapidly in a water bath 40°C. It should be rapid and is clearly dependent on the thickness of the container. For those two reasons the blood should be confined to a suitable shape to encourage heat transfer. Meryman and Hornblower (1971, 1972) found that plastic hemoflex 0.003-0.004 inch thick took six min., whilst bags of PVC 0.075 inch thickness require 10 minutes. They also recommended the minimum of handling or shaking since any squeezing of the contents in the presence of unthawed blood only exacerbated mechanical stress and haemolysis.

With careful attention to such detail on 314 consecutive occasions they achieved a post thaw recovery of 99.38% ± 0.44 - surely the highest ever likely to be achieved in any system (see also Pert 1969 for similar figures).

PVC bags as recommended by Huggins method are thawed in a water bath at 35°C to 43°C. Speed of thawing does not appear to be critical in this method just as speed of freezing is not critical.
Rowe et al (1970) recommended thawing with gentle agitation of blood filled flat stainless steel cans in their low glycerol frozen cells.

4. Post Thaw Washing Phase and Methods of Deglycerolization.

When red cells which have been stored with glycerol are to be transfused, it is necessary to reduce their glycerol content to about 2%, otherwise they will haemolyse on contact with plasma (Chaplin and Veall, 1953; Chaplin et al 1954).

Numerous methods have been proposed to remove the cell penetrating additive glycerol.

Mollison and Sloviter (1951) removed glycerol for thawed cells by dialysis. This was slow and cumbersome. Lovelock (1952) proposed a washing technique using hypertonic citrate to crenate the thawed cells thus removing intracellular glycerol by osmotic squeezing. Brown and Hardin (1953) advocated multiple washes in successively more dilute solutions of glycerol to remove the additive. Sloviter (1956) developed a controlled dilution procedure that did not require that glycerol be removed completely before transfusion. On the basis that human red cells are much less permeable to glucose than to glycerol, hypertonic glucose was added to the thawed erythrocyte/glycerol mixture to provide an osmotic counterbalance to the intracellular glycerol. Stepwise dilution of this mixture with an isotonic saline solution will not then cause haemolysis. Mechanical semi-automatic means of glycerol addition and removal have been introduced by various authors. (W/7 to 1/9). Tullis et al (1954) adapted the Cohn Blood Fractionator to add and remove glycerol. Cells in 50% w/v glycerol could be stored at -60°C and -120°C for up to five years and deglycerolization of the thawed mixture was done in a closed sterile continuous centrifugal technique using 10% glycerol followed by hypertonic sodium lactate and finally buffered isotonic saline solution with resuspension in 5% albumin or autologous plasma. Disposable plastic bowls were later adapted to standard centrifuges to allow efficient continuous deglycerolization under direct

vision (ADL), later to be known as the Haemonetics Bowl Centrifuge System (q.v.).

In 1963 Huggins (1963, 1965) introduced a novel way of removing dimethylsulphoxide, a penetrating additive, from large volumes of red cells that had been slowly frozen to -85°C and slowly thawed. This technique of "reversible agglomeration" allows the removal of additive by dilution with large volumes of non-electrolyte solution (8% glucose and 1% fructose). The low ionic strength allows the cells to undergo spontaneous clumping and these can be disaggregated by addition of electrolyte solutions. He later (1965) adapted his method of washing to remove glycerol from cells thawed from -85°C previously stored in 50% glycerol (5.4 moles/litre).

To avoid the considerable amount of washing needed to deglycerolize the red cells in the high glycerol slow freeze method Pert (1965) recommended a method of freezing in the presence of low glycerol concentrations and rapid freezing under controlled conditions in liquid nitrogen. A final glycerol concentration of 17.5% (w/v) with freezing at 144°C/minute gave best in vitro recovery (98%) in a wide variety of freezing containers. Washing the thawed glycerolised cells was achieved with 500 ml of 30% sucrose followed by 2000 ml isotonic saline. Initially this deglycerolization was achieved using serial centrifugation and later by using the modified ADL plastic bowl centrifuge system. Recovered cells can be resuspended in 5% albumin in saline or in plasma. Concern was expressed over the use of hypertonic sucrose, since it has been reported to produce renal damage (Anderson and Bathea 1960).

Krijnen et al (1964) reported the use of low glycerol rapid freezing of whole donations of red cells in liquid

nitrogen. Washing was achieved with hypertonic sorbitol to set up an osmotic gradient and avoid haemolysis in the first step of deglycerolization. This sugar is metabolised as quickly as dextrose and much more rapidly than sucrose and is therefore safer to administer to man. (Cohn and Gardiner 1962).

In a study of the high glycerol method Meryman (1968) found it extremely difficult to effectively deglycerolize cells without prior dilution. This is a particularly vulnerable time for the glycerolised and glycerol suspended thawed cells when the diluting solution must not be at such an osmolality as to cause excessive hypotonic stress on addition to the cells. Since glycerol at 4 or 5 Molar is approximately 25 times isotonic and cells can nearly double their water content before haemolysing, Meryman and Hornblower (1971) considered that a diluting solution of not less than half the osmolality of the glycerol was necessary. This initial dilution also increases the extracellular solute concentration, and so tends to reduce the cell volume. This helps to make the cells more resistant to swelling and more able to resist endosmosis and increase their density. They recommended 12% sodium chloride for the first dilution to give a final salt concentration of approximately 3.4% in the glycerolized post thaw suspension. In this way the cells lose about 70% of their free water although the bathing extracellular solution is approximately 20 times isotonic. This was calculated to mean that the cells could then be transferred to a solution 1/3 osmotic strength without swelling to bursting point. This was at 2.5 times isotonic and it was found that addition of 1 litre of 1.2% saline to

800 ml cell suspension would reduce the glycerol concentration to 1.4M and the sodium chloride to 2.1%. The final stage of washing is done in the ADL - Latham centrifuge washing apparatus by continuous infusion of 2 litres of isotonic saline (200 ml/minute) (see p80). Such a technique carefully performed again gave very high yields of 96.8% representing approximately 5 ml loss of starting cells in the bowl and tubing, - a truly remarkable achievement resulting in a final glycerol concentration of 0.125% (0.015M), a mean supernatant haemoglobin of 39.7 mgm/100 ml (250 units) ± 35.3 and no significant change in intracellular sodium or potassium.

Following the work of Rowe et al (1971) it was finally recommended that glucose should be added to the final suspension medium to help restore the energy utilizing systems and extend the shelf life beyond 24 hours but Valeri (1971) doubted this and stated that a decrease in cell potassium and increase in supernatant haemoglobin occurred during the post thaw storage at 4°C when resuspended in a glucose medium. Certainly for the low glycerol rapid freeze method using stainless steel cans or plastic bags as freezing vessels, Rowe et al (1970) recommended resuspension of the final washed recovered cells in 5% albumin, saline or glucose/saline. (p182).

In another wash system subsequently to become known as the Elutramatic counterflow centrifugal system, Schultz and Bellamy (1971) described the deglycerolization of frozen thawed red cells. In this machine the electronic programme unit sensors register the various parts of the cycle pre-

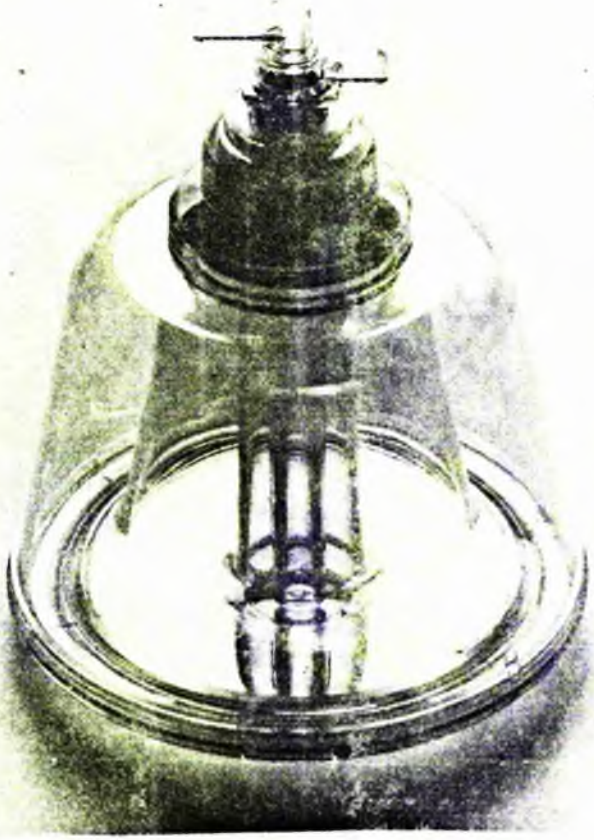
chosen by the operator. Apart from describing the various parts of the equipment no details are given of just how efficient the washing phase is with this machine. Orlina et al (1972) reported on the developed model to be known as "Elutramatic" (Fenwal Laboratories, Morton Grove, Illinois). In this system there is perhaps the highest loss of red cells of any recovery method (see Table 4/15, pt 4).

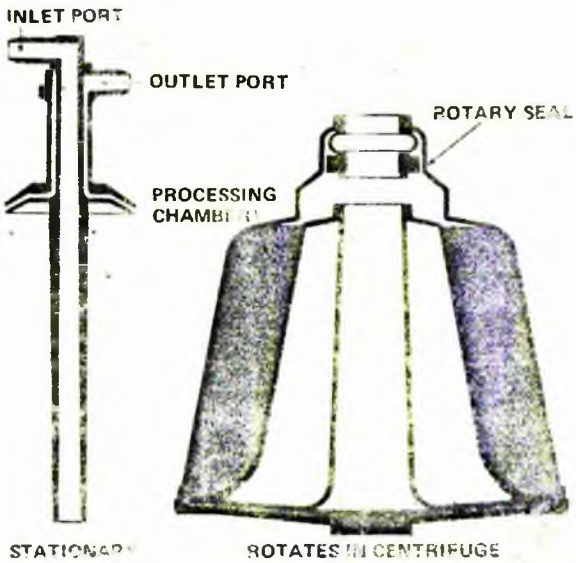
Deglycerolization is the main problem in all glycerol frozen cell techniques for the reasons already given. There are three basic methods now available.

(a) Removal of glycerol by Continuous Centrifugation.(Plate 1/7). Glycerolized red cells whether frozen in high or low glycerol concentration can be cleared by a continuous centrifugation principle using a special washing centrifuge bowl developed by Tullis et al (1956), Haynes et al (1960), and Tullis (1967), Tullis, Tinch and Latham (1971). The thawed glycerolized cells are transferred into a spinning bowl and are washed continuously by exposure to a gradient flow of hypertonic electrolyte solutions, followed by a final isotonic, electrolyte medium. The initial concentration of the thawed glycerolized red cells, the spindle speed of the bowl, and the density and tonicity of the wash solutions as well as their flow rates are all factors which determine the volume of the wash solutions required, and the time necessary to reduce the final intracellular glycerol concentration to less than 1% and the supernatant haemoglobin to acceptable levels of

250 mgm/100 ml - Valeri (1971). Valeri et al (1969) noted the value of prediletion with hypertonic salt to a final concentration of 5% glycerol as an initial step before centrifugation. The disadvantages of this method are the initial cost purchase of the semi-automated centrifuge equipment and associated transfer lines and disposable polycarbonate bowls, the relative slowness since it only does one unit of blood at a time making it no faster than manual batch washing where more than one unit has to be recovered. Unless a laboratory were to go in for large scale recoveries for considerable numbers of patients then inevitably the machine would sit idle for most of its life (Mitchell,1975). Conventional centrifuges have many other uses in the blood fraction programme and are frequently used for a variety of purposes. Staff training and operational needs are therefore minimal. Recently manufacturers have designed a larger version of the machine (Model 30) which has versatility for deglycerolization, plateletphoresis, leucophoresis and plasmapheresis but this is even more expensive than the Model 15. Tullis et al (1971) considered that the high glycerol method allowed a wider margin for error in the storage temperature providing a significant safeguard against temperature failure (due to mechanical deep freeze failure). They describe the ease of running their system of deep freezers at -80°C with storage in "supermarket" style run by two members of staff. They developed a polycarbonate disposable bowl and a brass non-disposable bowl together with all disposable

plastic non-reusable recovery kits. Reference to the diagram (Plate 1/1) shows that the centre sub-assembly remains stationary whilst the outer sub-assembly rotates about a veritcal axis. Rotary seals are provided between the two sub-assemblies. The original continuous centrifuge for deglycerolization was introduced as a development of the Cohn Blood Fractionator itself operating on the cream separator principle developed by the Protein Foundation Research Laboratories, later to become the Blood Research Laboratories, Boston Massachusetts (Haynes et al 1960; Sproul et al 1965; Valeri 1965, 1966). This proved to be too awkward and cumbersome and its cost and relative complexity hampered its development for routine use. Tullis and Tinch (1968) reported the development with the Arthur D. Little Company of stainless steel and plastic polycarbonate bowls to fit standard refrigerated centrifuges. Tullis et al (1967 and 1968) reported the development of an automatic cycle pumping unit with polycarbonate disposable bowls now widely known as the Haemonetics System (Haemonetics Inc.) (Tullis 1968, 1971 a,b; Meryman 1968, Pert 1969; Tinch et al 1971; Tullis, Tinch and Latham 1971; Pert 1971). Runck and Valeri (1972) however did caution that complete counterflow might not always be achieved within the bowl. Akerblom and Hogman (1974) assessed the Elutramatic Ultra-Flow system for the washing and recovery of their frozen cells from a low glycerol-liquid nitrogen bank first described by Orlina et al (1972) for use with Huggins high glycerol $-80^{o}C$ systems. The machine consists of a control centre for pumping and distribution of fluids, a refrigerated





The bowl has two parts, one that rotates, one that is stationary.

Plate 1/7. Haemonetics Bowl Washing Centrifuge.

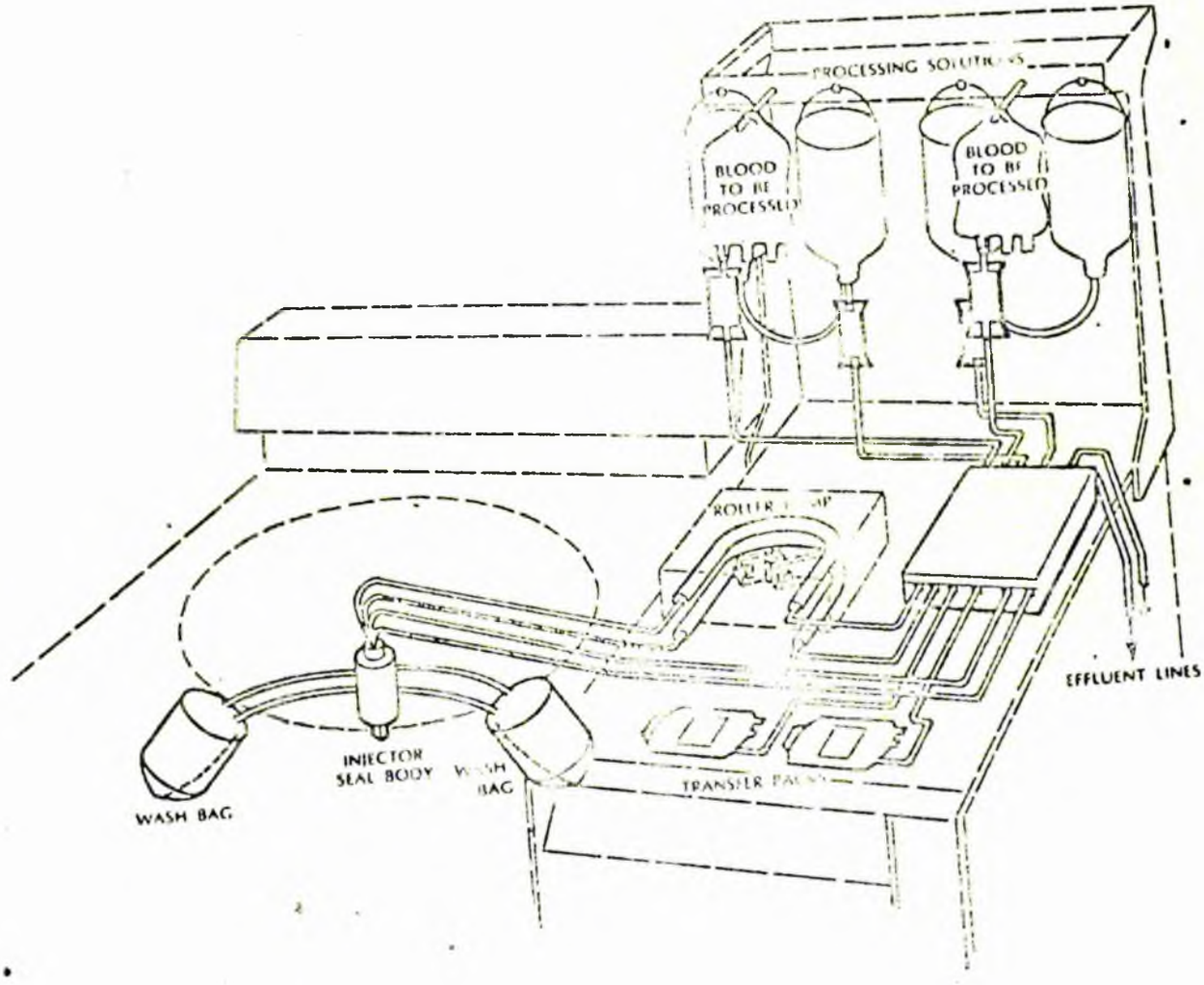


Plate 1/8. Fenwal Elutriamatic Wash System

centrifuge and a disposable set of plastic flow tubes, (Plate 1/8) wash bags and transfer packs. Two blood units are processed simultaneously. The blood wash solutions pass a fluid distribution plate controlled by photocells and are pumped into the wash bags of the centrifuge where the cells are processed by countercurrent continuous flow washing. Finally the washed red cells are pumped into the transfer packs. They commented on the amount of training and skill required to operate this system and the sometimes unexpected residual haemolysis in the final product. In general, the in vitro recovery was low at about 85% although 91.6 $\pm$ 1.8% in vivo survial of these recovered cells was noted. Biochemically the ATP and DPG levels were maintained as though in fresh blood but there was a 20% loss of intracellular potassium - a finding similar to that found for agglomerated cells recovered from high glycerol -80^{o}C banks (Runck, Valeri and Sampson 1968). (For review of machines see Goldman and Lowenthal 1975).

(b) Removal of Glycerol by Spontaneous Agglomeration.

The phenomenon of spontaneous agglomeration of red cells occurring in low ionic medium (Huggins 1965, 1966) is also used for washing high concentration glycerolized red cells. Washing is accomplished by sequential dilution with non-electrolyte solutions, the red cells being recovered by massive clumping and sedimentation (agglomeration). The non-electrolyte solutions used for washing and the volume and composition of the electrolyte solutions used for disaggregation are critical in achieving acceptable post transfusion survival of the red cells. Valeri et al (1966, 1967;), Runck, (1968), Akerblom et al (1968). The disadvantages

of this method are again the considerable expense in purchase of the special agglomeration machine designed to accept only a special PVC bag sold by the manufacturers. Large volumes of wash solutions are required (> 6 litres) and considerable losses occur in transfers and in the post thaw period where cells are not as stable. Overexposure of cells to the first contact with 50% dextrose (introduced as "osmotic balancer" - see p. 77) produced significant reductions in ATP levels (Lionetti et al 1966). Coombs (direct antiglobulin) positive erythrocytes were described after the initial recovery which persisted in vivo in some of the earlier work (Valeri 1966). This observation paralleled the discovery by Mollison and Polley (1964) that red cells in a low ionic medium acquired a coating of the C_3 component of complement and gamma globulin. This persists in vivo and although there is no evidence of diminished survival (Valeri 1966) minor matching difficulties do occur. With the low ionic strength media there is a loss of cellular potassium (Runck, Valeri and Sampson 1968; Valeri, Runck and Sampson 1969). Finally a centrifugation is needed at the last transfer to remove the low ionic medium and the 250 ml and saline needed to deagglomerate the cells. (Valeri and Rand 1966). In later work Ethylene diamine tetracetic acid was added to the glycerolization phase to prevent the coating of the cells, (Huggins 1966) but this resulted in processing losses of approximately 25% (Syzmanski and Valeri 1968; Almond and Valeri 1967; Moss and Valeri and Brodine 1968; Daane, Valeri and Barton 1969;, Valeri 1968) as well as in vivo loss of viability with only

85% survival 24 hour after infusion. (Valeri 1969). Storage for more than two years at -80°C (Valeri and Runck 1969) results in poor recovery probably because the low ionic medium is deleterious. Glycerol by contrast in an ionic medium gives satisfactory storage for at least 7 years. The addition of Na_2 EDTA although it prevents the Coombs positive phenomenon nevertheless adversely affects both in vitro and in vivo recovery and survival measurements especially after 1½-2 years storage in the frozen state at -80°C probably by binding calcium and magnesium in the red cell membrane. Washing with low ionic strength wash solutions such as recommended may further aggravate the condition. These limitations on the Huggins method suggest that washing should be done with ionic solutions and some have recommended electrolyte solution washing following pre-dilution with electrolyte (12% NaCl) or transfer of the agglomerated cells to a bowl washing centrifuge with further ionic washing in order to ensure improved recovery of cells, increased level of cellular potassium, decreased level of supernatant haemoglobin and improved 24 hour post transfusion survival (Tullis et al 1967; Demerjian and Kliman 1972; Runck and Valeri 1972; Orlina et al 1972; Gibson et al 1972; Meryman and Hornblower 1972; Meryman 1968).

(c) <u>Removal of Glycerol by Discontinuous Centrifugation (Batch Washing)</u>

Batch washing of low concentration glycerolized cells is carried out by serial centrifugation (Krijnen et al 1964, 1968; Rowe 1968). The glycerolized red cells are first diluted with hypertonic (salt) solutions then

centrifuged and the supernatant removed. They are then washed twice more with isotonic saline solutions, each time involving centrifugation and decantation of the supernatant. (see Methods in Technical Appendix).

5. Efficiency of Washing Processes - Post Thaw Stability

As well as consideration of time, number of transfers, ease of use of established programming and reproducibility when continuous flow washing is used as opposed to intermittent batch washing others have attempted to assess these two in other ways. Button et al (1972) described findings in preparations of washed cells taken from conventional bank to render them suitable for patients undergoing haemodialysis, awaiting organ transplant or who have recurrent non-haemolytic febrile reactions to transfusion. They found that cells washed by machine survived better in vivo, residual plasma was diluted 1/25,000 by machine washing compared to 1/600 by manual washing.

Some interesting experiments with donations inoculated with hepatitis virus (HBsAg), cytomegalovirus (CMV). Poliovirus type I (PVS) have been reported. In both machine and manual techniques no viruses were detectable in the final product. In the manual method no viruses were detectable beyond the second wash. Plaque assay or monolayer culture of African green monkey kidney cells were used for Poliovirus; human fibroblast culture with and without neutralisation by specific anti-serum was used for CMV, and immunodiffusion, complement fixation and radioimmunoassay for hepatitis virus.

White cells were difficult to remove by machine washing

and by batch washing. In 20 random samples of batch washed red cells they found on average 8,825 leucocyte/ cub mm. Differential counts showed a selective loss of granulocytes with retention of lymphocytes. This was attributed to the lymphocyte being similar to red cell specific gravity (Wintrobe) with a tendency to pack with the red cells.

Although the infectivity titre of HBsAg fell from 1/32 to undetectable in the final washings of the injected blood they point out that the most sensitive techniques (Radioimmunoassay) are still far less sensitive than infectivity in man (Wallace 1976) and the absence of detectable HBsAg cannot be accepted as evidence of safety, especially if, as in the case of CMV, the infection is probably transmitted via the leucocytes which are only reduced at most by about 50% confirming Kliman's study with the ADL centrifuge.(1972)

Before agreeing however that machine washing is better than batch washing it is important to notice in Table 2 of these authors that in fact they were not comparing like with like, since in the machine method they used 2 litres of wash solution as against only 1 litre in the manual method.

It has also been noted by Runck and Valeri (1972) that it is not always possible to achieve true mixing within these bowl centrifuges and that a proper "counter current" method of mixing cells and fluids travelling in opposite directions within the bowl does not always occur (Tullis et al 1967; Meryman 1966). This places difficulties over the optimal flow rates and hence the

washing time especially with high glycerol freezing methods based on measurements of residual Evans Blue dye left in the bowl and residual glycerol left in the bowl.

Demerjian and Kliman cite Meryman's (1972) advice that the bowl washing centrifuge results in a plasma dilution of approximately 1.2×10^{6}. Meryman himself (1968) stated that the ADL system gave a reduction in concentration of any extracellular contaminant by a factor of 100 in less than 5 minutes when the cells were suspended in isotonic saline but introduction of glycerol considerably impaired this. In assessing the efficiency of batch (manual) washing it is useful to remember that centrifuging requires less than a litre of wash solution. Despite Valeri's claim (1971) that batch washing takes 2 hours this must be repudiated. Although he says that for large scale clinical use batch washing is too time consuming and laborious he does not define large scale use nor the technical help needed to achieve the washed cells. In our laboratory we now have a fairly busy frozen cell bank where an average 3 or 4 units are being recovered each working day. On some days up to 8 have been recovered without undue strain despite the fact that the processing is done by a laboratory assistant who either works alone or has a learner helper who can attend to the housekeeping chores of keeping stocks of reagents and equipment - transfer packs, needles, tubes etc. up to date and cleaning up after a recovery. The whole cryogenics section is now under the control of a Chief Technician who was appointed in Phase 2 of this work (September 1975).

B. Low Concentration Glycerol - Rapid Freeze Methods.

These methods have been described by Pert, Schork and Moore 1964; Pert, Moore and Schork 1965; Krijnen et al 1964; Rowe, Eyster and Kellner 1968; Jenkins and Blagdon 1971; Mitchell et al 1972. The low glycerol, (17%-19%) rapid and easy recovery method has advantages over high glycerol systems (Runck and Valeri 1969). The dimensional problems of container design have been overcome by using either stainless steel flat containers 8 mm thick (Rowe 1967, and see p. 70) or Fluorocarbon laminate plastic bags (Teflon-FEP) of 3 mm thickness which can withstand the lower temperature of liquid nitrogen (Pert et al 1965). Less than 1 litre of wash solutions are required in the serial centrifugal washing of the thawed cells to achieve a very efficient deglycerolization (0.40 gm/dl $\pm$ 0.1 for batch washing, 0.26 gm/dl $\pm$ 0.1 for continuous bowl washing) (Valeri, Runck and Brodine 1969). No significant changes occur in cellular potassium although glucose/saline resuspension medium resulting in some leak did occur on subsequent storage at $4^{o}C$ as well as some additional haemolysis.

These methods, since they use liquid nitrogen for freezing and storage, require either metal (aluminium) or Teflon containers compared to the high glycerol $-80^{o}C$ methods which need only PVC containers. Batch washing is said to take up to 2 hours (Valeri 1971; Meryman and Hornblower 1971; Pert 1969) but at least more than one unit can be manually processed at a time unless one is prepared to buy a battery of processing machines to allow simultaneous multi-unit recovery!

These methods also get round the technical problems of how to wash away high concentration of glycerol and yet provide a sterile product whose recovery is adequate and whose in vitro survival is acceptable with no demonstrably deleterious effect on the cells (see p.148).

Akerblom et al (1968) attempted to combine the rapid freezing method described by Pert et al (1965) and Krijnen et al (1964), with an agglomeration washing procedure like that described by Huggins (1964, 1965). The agglomeration procedure requires a low ionic strength (q.v.) whilst a rather high salt concentration is needed for the freezing. By suitably modifying and adapting the preserving and wash recovery fluids it was possible to modify the low glycerol rapid freeze method to obtain recoveries of satisfactory cells by either batch washing or cytoagglomerating cells stored by a low glycerol rapid freeze method. (approx. 16% glycerol w/v). These cells all have a post transfusion survival of more than 87%.

Pert and Schork (1969) reported on the use of the ADL bowl washing centrifuge method of freezing thawed cells of their residual glycerol by countercurrent use of sucrose or mannitol solutions. Since sucrose is subject to hydrolysis it is recommended that sucrose in the final wash should be avoided to prevent excessive haemolysis.

Post Thaw Stability.

In an effort to prolong the shelf life of thawed blood Gibson et al using the high glycerol method and automatic washing by machine investigated this problem. Their results are shown in Table 1/2. These authors felt on this basis that washed thawed red blood cells suspended in autologous plasma would give satisfactory results for at least 14 days storage post thaw recovery at 4°C for CPD collected donations and 7 days for ACD donations. It is to be noted that although cells resuspended in plasma may remain vialbe for a longer time post thaw, the claim that frozen cells lacking plasma white cell and platelet antigens are not likely to allergic reactions is void under such circumstances (Valeri 1966).

Enzymes of red cells maintained at 4°C retain activity for weeks beyond the time when the cells no longer demonstrate acceptable in vivo survival. This applies to all of the glycolytic (energy producing) enzymes for 5-6 weeks at 4°C. Subsequently some reductions occur - hexokinase 38%, phospho fructokinase 96% and pyruvate kinase 62% (Tullis and Lionetti 1966). Freezing preserves the enzymes. When previous frozen deglycerolized red cells are suspended in their original plasma and kept at 4°C their metabolic characteristics are very similar to those of unfrozen blood. However many degenerative changes are accelerated and at 4°C the post thaw storage time is therefore only about 1 week (Tullis and Lionetti 1966).

Valeri (1965) showed that reconstituted blood will give acceptable post transfusion survivals after storage at 4°C for up to 14 days but the best results are obtained

TABLE 1/2

Post Thaw Stability (after Gibson et al 1972)

Measurement		Control	0	5	10	5
MCV	ACD	93.2	96.5	99.5	99.8	102.8
(μ^3)	CPD	92.0	90.0	91.5	90.8	90.5
pH	ACD	6.84	6.98	6.87	6.77	6.67
	CPD	7.06	7.22	7.04	6.87	6.73
ATP	ACD	2.7	3.2	2.5	2.2	2.1 (1.58)*
µM/gmHb	CPD	2.8	2.8	2.4	1.9	1.5 (2.12)*
		1.05	1.01	0.80	0.58	0.40 **
Glucose	ACD	105	95	77	70	63
µM/gHb	CPD	113	86	64	46	36
Plasma Hb	ACD	3	136	161	198	253
mgm/dl	CPD	4	44	75	92	110
Supernatant		10	50	100	115	200 **
Potassium	ACD	5.5	4.7	14.7	19.8	20.8
meq/l	CPD	3.7	2.6	10.4	16.9	20.9
Supernatant		3.0	4.0	10.0	18.0	21.0 **
in vivo survival	ACD	87	87	-	63	61
% at 24 hrs	CPD	89	86	-	78	60
		97.5	76.0	(T/2 = 30 days) **		
		100	81.5	(T/2 = 30 days) **		
		97	81.5	(T/2 = 28 days) **		

* Rowe et al (1970) 14 days at 4°C post thaw + 12 hrs at 37°C.

** Prins and Loos (1965)

TABLE 1/2

by transfusion within 48 hours of reconstitution.

Valeri (1969) wrote of the poor in vivo survival of post thawed cells recovered by Huggins storage after 2 years and would only consider less than a 24 hour shelf life at 4°C because of this. In contrast blood recovered in ionic media by automated (Cohn) centrifugation from Huggins storage banks for up to 6 years gave satisfactory storage for at least 24 hours at 4°C. Similar, less exhaustive, figures were obtained by Orlina et al (1972) who used the Elutramatic system to wash frozen thawed cells laid down by a high glycerol slow freeze method (Huggins) using ACD collected blood. Huggins (1970) recommended a shelf life of 28 hours under normal circumstances thus permitting cells processed in the morning to be used until the following afternoon.

In the low glycerol method of storage with either batch or centrifugal countercurrent continuous flow washing Pert and Schork (1969) showed an initial loss of 10% in intracellular potassium due to freezing and thawing with a steady decline thereafter on storage in mannitol saline at 4°C till it had fallen to about 55% on the eigth day. Throughout this time supernatant haemoglobin rose by incremental haemolysis until it reached around 400 mgm on the 14 days storage at 4°C (200 mgm at day 8).

As well as washing away the glycerol, the various recovery processes described for deglycerolization are now known to have the added advantage of purifying the blood of unwanted materials e.g. leucocytes, platelets, plasma, cations, anti-coagulants and viruses, and anti-A

and anti-B antibodies. Thus removal of glycerol which was originally considered a disadvantage of the frozen cell recovery programme is now reckoned to be an essential part of the process which conveys a certain unique quality to the recovered blood. Reference to this will be made in the appropriate section later in this thesis - part III.

THE APPLICATION OF KNOWLEDGE TO BLOOD STORAGE BY FREEZING AND RECOVERY USING GLYCEROL

Although Smith (1950) described successful storage of blood in small amounts it was only to be expected that others would become interested in the storage of whole donations of blood. Mollison and Sloviter (1951), Chaplin and Mollison (1953), studied the use of glycerol and discovered that a high content of glycerol (40%) when mixed with an equal volume of packed red cells gave satisfactory preservation and recovery (98%). Brown and Hardin, (1953) similarly used glycerol in a two stage addition technique to a total final glycerol concentration of 44% and obtained a recovery of only 77% in vitro and 47% in vivo, 24 hours post infusion after storage at $-15^{o}C$. Storage at $-70^{o}C$ resulted in better recoveries (90%) in vitro but only 64% in vivo.

Chaplin et al (1954) confirmed the value of high glycerol concentrations (30% w/v) at $-20^{o}C$ and later (1956) showed the best results at $-79^{o}C$ (90% in vitro recovery). Since high concentration of glycerol can of itself damage cells and cause excessive haemolysis (Hughes-Jones et al 1957; Mollison 1956) it is important to obtain rapid mixing of cells and cryoprotective during the preparation stage or a two stage addition of diluent must be used (Schmidt and Steinfeld 1960). The temperature of $-79^{o}C$ is relatively easy to maintain (mechanically or by solid CO_2) but high temperatures have been tried but with resulting lower proficiency of recovery depending on how near the ambient storage temperature is to $-35^{o}C$ which

Lovelock (1953) showed was the most damaging for salt lysis (see p.35). From these studies arose the high glycerol slow freeze methods made popular in U.S.A. by Ketchel et al (1958), Tullis et al (1958), Haynes et al (1960), O'Brien et al (1961), Huggins (1963, 1964, 1966), Huggins and Grove-Rasmussen (1965).

Due to the rapid ingress of water into glycerolized cells resuspended in isotonic media (p.51) it is necessary to remove glycerol before transfusion. This can be achieved by dialysis, osmotic squeezing or batch washing in continuous or discrete washing cycles using gradually diminishing amounts of glycerol or a combination of these. Because of the long time required for dialysis and the difficulty in maintaining sterility this method has been superseded so far as preparation of large volumes of cells is concerned. The principal washing techniques are:

1. Glycerolised red cells, whether frozen in high or low concentration of glycerol can be washed using a continuous centrifugation principle with counter-flow of cells in one direction in a washing bowl against a flow of wash solutions in the opposite direction. The thawed glycerolized cells are transferred aseptically and washed by continuous hypertonic electrolyte solutions followed by a final isotonic electrolyte medium. The initial concentration of the thawed glycerolized cells, the density and tonicity of the solutions, the spindle speed of the bowl and the flow rates are factors which determine the volume of wash

solution and the time recovery to reduce the final intracellular glycerol concentration to less than 1% and the supernatant haemoglobin to an acceptable level (p156).

2. The phenomenon of spontaneous agglomeration of red cells occurring in low ionic medium as introduced by Huggins (1965), is also used for removing high concentration of glycerol from red cells. Washing is achieved by sequential dilution with non-electrolyte solutions, the red cells being recovered by massive clumping and sedimentation (agglomeration). The non-electrolyte solutions used for washing, and the volume and composition of the electrolyte solutions used for disaggregation of the agglomerated cells are critical in maintaining acceptable post transfusion survival (Valeri et al 1967, 1968, 1969). Moreover the exposure of red cells to a low ionic environment produces a significant loss of potassium from the cells (p180).

3. Batch washing of low concentration glycerolised red cells is carried out by serial centrifugation. (Krijnen et al 1964; Rowe et al 1968). The glycerolised cells are initially diluted with hypertonic salt solution, following which they are centrifuged for packing, and the supernatant fluid removed. The red cells are then washed twice more with isotonic saline solutions, each time involving centrifugation and decantation of the supernatant.

Lovelock (1952) introduced the idea of osmotic squeezing using sodium citrate addition to the cells in suspension. Sodium citrate does not penetrate the cells and so water and glycerol are 'wrung' from the cells and can be then transferred to buffered saline or

plasma without too much haemolysis. Glucose has a similar effect (Sloviter 1956; Sloviter and Ravdin 1965). Hypertonic sugar solutions - 10% sorbitol were chosen by Krijnen as a first wash for cells stored in 17.5% w/v glycerol in liquid nitrogen after intermediate freezing in liquid nitrogen (p68). Sorbitol was chosen since at the time of the original choice, sorbitol was licensed in the Netherlands and so the choice was made, in order not to impede early introduction of the sugar in patient care (Krijnen - personal communication). This is the basis of the method described in this work.

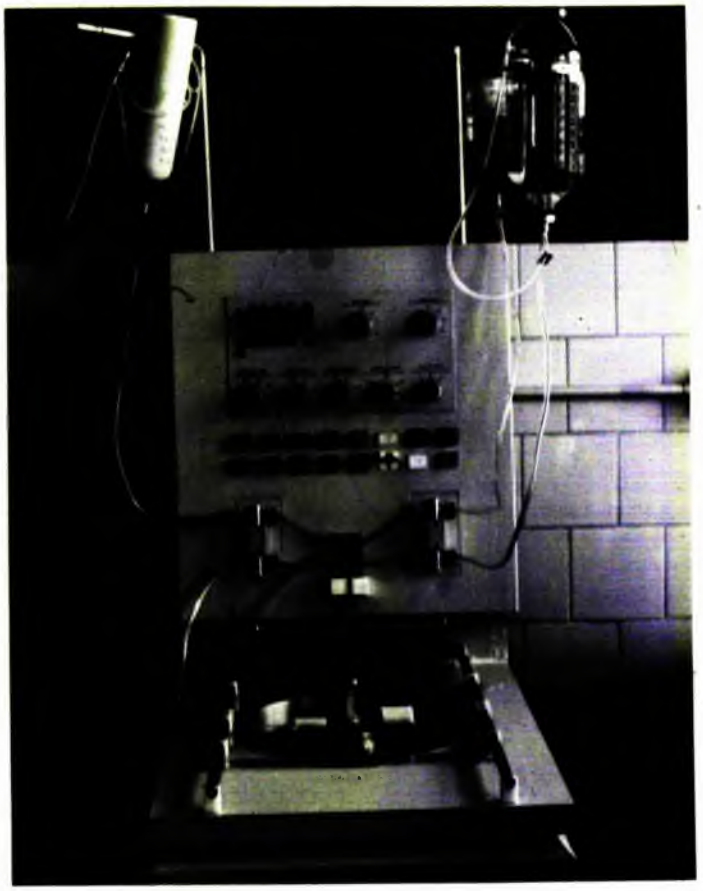
Huggins (1963) described washing glycerolised cells by 'reversible agglomeration' - diluting thawed cells in 10% glucose which causes sludging. This can also occur in any system where cells are suspended in large volumes of electrolyte free sugar solutions, such as occurs in a transfusion set when red cells are wrongly mixed with dextrose solutions. Cells under the influence of low ionic strength medium rapidly agglomerate. Thereafter supernatant can be removed and replaced with further washing solutions which if they contain electrolytes will reverse the process of agglomeration (e.g. saline or plasma) (see p 78).

4. Combined methods of deglycerolisation have been developed to speed up the rate of processing. Generally these offer a direct removal of supernatant containing glycerol and its replacement with gradually diminishing quantities of glycerol as further wash

solutions are brought in. This method is often combined with the use of a hypertonic sugar in the first wash solution to further squeeze the cells and hasten the deglycerolisation. This is the basis of the present batch wash cycle methods used in many laboratories and the one used on the present study (Krijnen 1964, 1968; Jenkins and Blagdon 1971). Further refinement of techniques have resulted in continuous automatic cell washing centrifuge in which the wash solutions are applied as a continuous stream percolating through the centrifuge mass of cells. The glycerol rich supernatant is carried off to waste. Such early machines were developed by Chaplin and Veall (1953), Mollison et al (1958), Tullis et al (1958), Haynes et al (1960), Sproul and Zemp (1962). Some excellent modifications of these systems have been developed in recent years with a more elegant electronic control of the operating conditions for each machine (see p100). Thus Travenol Laboratories have expanded the original Mollison et al method of continuously washing two bottles of 550 capacity using a standard centrifuge to a sophisticated (Elutramatic) assembly of plastic bags, plastic tubing and plastic disposal wash bags and collection bags. Flow rates within the machine, centrifuge speeds and time can all be independently varied and electronic devices sense the supernatant haemoglobin level to ensure adequate washing to a predetermined amount. The machine and its equipment however are very expensive.

The author has seen such machines in Brussels, Amsterdam, Stockholm, Uppsala and London but on each occasion the machine has not performed as expected and on each occasion 'minor' adjustments were required with the electronic circuitry. When the author visited Amsterdam transfusion centre in 1970, Mr. George Judson with the local staff was testing the prototype of the IBM machine in which a special plastic bag of circular shape is mounted on a flat plate turned by a centrifugal rotor. An inflatable bag beneath the blood bag can be used to pressurise the blood bag contents and express them via a central outlet line which also serves as inlet for wash solutions. This machine is again complicated by the amount of electronic control gear which on the basis of supernatant haemoglobin can vary the centrifuge speed, the flow rates and time taken to complete the cycle. The circular bag looks a little strange when hanging above a patient's bed but the system has been found to be acceptable when used as the wash system for blood stored by the method of modified Krijnen in aluminium cans as detailed by Jenkins and Blagdon (1971). 98% recoveries of viable red cells can be achieved in routine use (Winnick 1974 - personal communication)(Plate 1/9). and the cells are acceptable at clinical level in hospitals supplied by Army Blood Supply Depot. Two further refinements to the use of continuous centrifugal washing are the Amines and Haemonetic bowl washing centrifuges. A report by the author presented to

Filling the Machine



Wash solutions in position

Plate 1/9. IBM 2991 System

Separator
in Action

Final Product
for Issue

Scottish National Blood Transfusion Service on these machines is included in the Appendix as part of this thesis. Basically although the idea of these machines is attractive their high cost and the fact that they must of necessity remain idle for most of the time precludes their routine use in the field of frozen cells (Mitchell et al 1973). At the time of writing the polycarbonate individual bowls of the Haemonetics bowl washing centrifuge are £12.50 each and the harnesses (flow tubing) and solutions account for another £3. Even allowing for the use of a bowl to wash two pints of frozen cells the cost of the blood is doubled before reaching the patient. In the present economic position such expense is unjustified for such a simple task which can be just as efficiently performed by batch washing using discrete centrifugation. The necessary centrifuges and plastic transfer bags, adapters etc. are readily available in most large transfusion laboratories. The wash solutions are not expensive and most technical staff can handle the simple manipulation required since the basic skills are well developed at this level. These same skills and the centrifuges can be used for many other tasks in a busy department and in my view fully justify the adoption of the batch washing principle. The idea of using electronic washing machines with press button operation clearly appeals to some who nevertheless use technologists for the work. Where technology is not advanced or is unreliable then the use of highly sophisticated

machines which require expert maintenance and servicing is not going to solve the difficulties.

All of these mechanical automated methods of recovery have sought to improve on the existing washing procedure. In this they have only been partially successful and their great cost must make one exercise caution before their uncritical introduction.

For the same reasons other forms of extremely expensive automated centrifuges such as the IBM celltrifuge or the Haemometres Model 30 cannot be justifiably used to merely deglycerolize frozen thawed blood. These machines, of course, were introduced for other aspects of patient care such as providing high yields of leucocytes and platelets to support patients with temporary bone marrow suppression. For authoritative review see Goldman and Lowenthal (1975).

PART II

THE PROBLEMS OF INTRODUCING A FROZEN CELL BANK.

DESIGN OF STUDIES ON FROZEN CELLS.

"The frost performs its secret ministry
Unhelped by any wind".

S.T. Coleridge, Frost at Midnight

I mention, not after the best manner that might be, but after the best manner, that was practicable by the accommodations I was then able to procure: so that it need not be wondered at, or blamed, if in some passages of these Papers, Experiments to the self same purpose are more accurately tri'd, or by more Expedient ways at one time than another. For as a Physician, if he come to practise in the Country, where Apothecaries shops are but ill furnished, both as to the Number and as to the Quality of the Drugs, must accommodate his Practise to the scant Materia Medica, of which alone he has the command: So when I write of Experimental matters, in places where I cannot have Workmen, nor Instruments fit for my turn, I must be content to vary my Experiments accordingly, and fute them to the accommodations I am confin'd to, which, though it be an unwelcome Condition, is made the less so to me, by a Hope, that the Equitable Readers will think it to be all that a man is bound to do in such cases, to procure the best assistances he can, and manage those, he is able to procure, to the best Advantage.

And this I the rather take notice of on this occasion, that ingenious men might not be too much discouraged by imagining, that, because they live in the Country, or upon other scores cannot furnish themselves with the best Instruments and Accommodations, nor enjoy the assistance of the skilfullest Artificers; they are either unqualified for the making of Experiments and Observations, or Superseded from it. For though in some cases, where the measures of things must be nicely determin'd, and principally in Observations, whereon either Theorems *or* Hypotheses *about the* Proportions *of things are to be grounded; very good Instruments are exceeding useful, and sometimes necessary: yet there are Thousands of particulars, whose knowledge may be instructive to those, that Are or Would be Naturalists, where no such Nicety is requisite, and where the measuring things by* Ounces *and* Inches *will serve the turn, without determining them to* Lines, *and* Grains. *And even in cases, where Exact observations are (to some purposes) Requisite, those that are not so, may be sometimes very useful, by affording Hints, by which others may be excited and assisted to make those more accurate Trials. And here let me take notice, that a Tool*

or

"I mention, not after the best manner that might be, but after the best manner, that was practicable by the accommodations I was then able to procure: so that it need not be wondered at, or blamed, if in some passages of these Papers, Experiments to the self same purpose are more accurately tri'd, or by more Expedient ways at one time than another. For as a Physician, if he come to practise in the Country, where Apothecaries shops are but ill furnished, both as to the Number and as to the Quality of the Drugs, must accommodate his Practise to the scant Materia Medica, of which alone he has the command: So when I write of Experimental matters, in places where I cannot have Workmen, nor Instruments fit for my turn, I must be content to vary my Experiments accordingly, and sute them to the accommodations I am confin'd to, which, though it be an unwelcome Condition, is made the less so to me, by a Hope, that the Equitable Readers will think it to be all that a man is bound to do in such cases, to procure the best assistances he can, and manage those, he is able to procure, to the best Advantage.

And this I the rather take notice of on this occasion, that ingenious men might not be too much discouraged by imagining, that, because they live in the Country, or upon other scores cannot furnish themselves with the best Instruments and Accommodations, nor enjoy the assistance of the skilfullest Artificers; they are either unqualified for the making of Experiments and Observations, or Superseded from it. For though in some cases, where the measures of things must be nicely determined, and principally in Observations, whereon either Theorems or Hypotheses are exceedingly useful, and sometimes necessary: yet there are Thousands of particulars, whose knowledge may be instructive to those, that Are or Would be Naturalists, where no such Nicety is requisite." ROBERT BOYLE, FRS (1683)

APOLOGY ?

Plate 2/1

10 la.

THE INTRODUCTION OF A FROZEN CELL BANK

The history of frozen blood spans just over 25 years from 1950 to the present. The first ten years (the 1950's) were spent investigating and defining the problems of freeze-thaw injury to cells and there developed an empirical approach to its prevention which, during the next ten years (the 1960's), grew into a series of detailed methods for the collection and storage of viable transfusable red cells. During the 1970's experience has been gained so that it was felt that the time had come for Glasgow and West of Scotland Transfusion Centre to become involved in the further investigations and development of these methods and in doing so to determine which, if any, of the methods was the most practicable for transfusion therapy.

Accordingly the author at the request of the Regional Director in 1969 visited three important centres with a view to solving these problems - the British Army Blood Supply Depot at Aldershot where considerable experience had been accumulating since 1964, the Netherlands Red Cross Blood Transfusion Centre in Amsterdam where some European countries had been storing frozen donations from 'rare' donors and the Regional Blood Transfusion Centre at Brentwood, Essex, where Dr. John Blagdon with the help of the Army had recently set up a frozen cell bank with the aid of a DHSS grant.

The author wrote a full report of these visits (see Appendix) and these together with a study of the

USE OF FROZEN BLOOD - THREE PHASES

Phase	Number of Months	Units Transfused
08:11:71 - 31:01:72	6	25
01:02:72 - 31:01:73	37	305
01:02:73 - 31:08:74	154	736
TOTAL	217	1066

Total units frozen 1971-74 2110
units frozen in 1974 1026

Table 2/1 Three Phases of Development

literature made the foundation for an application to the Scottish Home and Health Department for a special development grant of £3000 for a feasibility study.

The history of the project since that time can be divided into three phases:- (Table 2/1)

Phase 1 where time was spent sifting literature and developing techniques together with making new observations on the technological problems.

Phase 2 where time was spent in introducing the method for clinical application and extension to certain important groups of patients.

Phase 3 where time was spent consolidating knowledge of the system, introducing technology to Glasgow Western Infirmary where renal dialysis and transplantation is carried out. Further expansion and advice has been offered to Glasgow Royal Infirmary who have also set up a small satellite frozen cell bank and to the Regional Transfusion Centres at Aberdeen, Inverness, Dundee, Leeds, as well as other hospitals such as Guy's Hospital, London. These phases will be dealt with in detail.

The Choice of Method. The rapid freeze-low glycerol method of freezing whole donations was chosen as the method of choice for the following reasons:-

(i) No expensive apparatus was required. A simple extruded one piece aluminium cannister with suitable gas tight sealed neck had been developed by Meijnen and his colleagues which at the time of initial enquiry

could be purchased in Britain for approximately £2.00. Glycerol was readily available and easy to handle by dilution and sterilisation without deterioration. It had been shown to be an excellent cryoprotectant in all methods and although it required removal before infusion of the thawed cells it in itself was not toxic and only required time for osmotic balancing of the cell contents to prevent abnormal haemolysis in vivo (see p 96). Smaller quantities, 19%,of glycerol were used in the rapid freeze method using liquid nitrogen as against mechanical refrigeration so that washing was easier. Liquid nitrogen was readily available from a local depot of the British Oxygen Company (Carfin, Motherwell). We were assured of continuing supplies of liquid at modest cost compared to the alternative of using a mechanical high performance and high cost deep storage refrigerator which could be used with a high (40%) glycerol method and slow freezing. Such a system was also subject to mechanical and electrical failure and it seemed too vulnerable to such interruption of temperature control with the threat of total stock loss especially since the temperatures likely to be encountered might quickly become perilously near to the -35°C range where it is known salt lysis and cell damage is maximal (see p30). The liquid nitrogen system had the additional merit that no mechanical or electrical power source was required for trouble free maintenance in the specially adapted large storage dewars flasks made of high quality stainless steel. Provided these containers were regularly inspected and topped up as required nothing

short of a catastrophic loss of superinsulation and skin vacuum in the dewars (really large thermos flasks) could result in abnormal rapid loss of nitrogen. Even then we were able to get an assurance from B.O.C. that in such an improbable event they would (a) provide a tanker of liquid nitrogen to continuously maintain the level of liquid nitrogen (b) provide a replacement vessel if one was not available locally to make a stock transfer and (c) provide a system of re-establishing the vessel vacuum if this was required. The liquid nitrogen system gave further advantages in that small volumes of liquid were already being handled in the department in a droplet freezing method using PVP as cryoprotectant. When it came to thawing the frozen cells and their deglycerolisation it was a simple matter to modify the Krijnen method to thaw the cells and transfer these to a special wash bag for centrifugation and batch washing (see p86). This was much easier than the high glycerol slow freeze method of Huggins where an expensive apparatus (on loan to ABSD) was needed to carry out the manoeuvres of reversible agglomeration (see p78). Despite the claims regarding the rapid through-put in a large frozen cell bank (Hurn 1971) it was not envisaged that such processing would be required and that cells would be recovered by special requests for planned transfusion in selected patients. The thawed cells from the liquid nitrogen system could be placed into the transfer bag costing approximately 50p and the whole centrifuged in a standard M.S.E. centrifuge, which of course, could be used for many other tasks in the blood fractionation programme and could be operated by staff already familiar with the operating instructions.

Although the Army were interested in developing a method of cryopreservation which did not require removal of the cryoprotectant, the methods using PVP and other macromolecular substances were known to give much lower yields of cells in the recoveries and cells did not survive as well in vivo (see p60).

On the basis of these findings and thoughts, it was decided to try to investigate the feasibility of introducing a small bank of frozen cells at the Regional Transfusion Centre at Law Hospital. In this I was particularly fortunate in having the assistance of Mr. William Muir, F.I.M.L.S., a Principal Chief Technician of long experience in blood transfusion techniques. When the project was discussed he willingly agreed to co-operate during all phases of the work especially in the early stages when we had to improvise in so many ways to ensure success.

Since the Krijnen Cannister was expensive and almost doubled the cost of a unit of blood Dr. John Blagdon (1971) had been successful in developing the use of a modified extruded aluminium can similar in basic design to the Amsterdam type although with a tightly secured rubber subaseal stopper wired in position. We obtained some of these from Mr. David Wilde, Marketing Manager for Spembly Technical Products, Sittingbourne, Kent. He had a basic interest in low temperature physics and cryogenics in particular. When the problem was put to him of an alternative to the Krijnen can he discovered the new one as a by-product of the aerosol hair-lacquer industry in Finland (Printaloy). The author visited Dr. Blagdon and

obtained samples of cans and rubber stoppers. Much of the credit for developing an operational can was therefore due to Dr. John Blagdon, who so willingly helped in the early stages of the work. Combining experience of his methods and those of Krijnen we quickly took the plunge into the first bath of liquid nitrogen not quite knowing what would happen! At that time we worked in a corner of the basement and equipment consisted of a 500 litre liquid nitrogen storage tank (TWN 500), and 250 litre liquid nitrogen storage Dewar (CPV 250), a battery operated pump delivering 2 litres of liquid nitrogen per minute. A smaller open-necked Dewar (ODS 18) was available in which the cans were stood singly during the static freezing (see Plate 2/2). Freezing of the cans presented a considerable problem and our first attempts consisted of making a 'flotation collar' cut from blocks of polystyrene foam sufficiently bouyant to enable the filled can to float in the liquid nitrogen but not over its neck since it was thought (and later proved correct.!) that liquid nitrogen entering the cans during freezing could present an explosion hazard during subsequent thawing since liquified gas vapourising would greatly expand its volume and rupture the cannister. This flotation collar proved very successful and served the additional purpose of allowing frozen cans to be thawed in a large volume of water at 40°C. Early experiments were very much an unknown quantity and I have a vivid memory of our first thawings in a water filled Woolworth's plastic dustbin in which a can with a slowly ballooning rubber stopper

Plate 2/2. Cryogenic Laboratory Equipment
pagellla.

bobbed about on the flotation collar whilst Mr. Muir and I kept observation from the safety of a nearby brick column supporting the roof! This flotation device formed the basis of an early paper described by us in J. Clin. Path. (see Appendix).

Having successfully frozen and thawed blood it was essential to proceed to (b) train more staff to operate the system, develop a proper system of recovery of the cells in a sterile environment with as little loss and damage as possible and in a fit state for transfusion (c) to establish a series of basic measurements on the product and (d) to establish a single effective inventory control system with careful and accurate information about each donation.

THE SYSTEM OF RECOVERY OF CELLS

(See Technical Appendix)

Krijnen developed a five tail bag into which the thawed blood is passed after thawing, by means of a needle and integral transfer line. Deglycerolization is performed in a stepwise removal first by removing the supernatant after centrifugation, second by introduction of hypertonic sorbitol solution to osmotically squeeze the cells free of glycerol and third by further removal of supernatant and introduction of a normal saline wash followed by centrifugation and supernatant removal and finally addition of a small amount of normal saline (70 ml) to lower the viscosity of the product and to issue a saline suspension of concentrated red cells virtually free of leucocytes, platelets, glycerol and cations except sodium. As previously stated these five tail bags (Plate 1/5-6) were developed by Krijnen and made available in U.K. but it was not long before Tuta Laboratories were able to manufacture a wash bag to a similar design with certain special advantages. The giving ports were mounted on the end of the bag and identical with already clinically acceptable blood bags. It was felt (i) that the everted umbilicus of the Krijnen bag was incompatible with British Transfusion infusion giving set piercing units because of the awkward angle of delivery and the dangers of metal tipped spikes or piercing needles penetrating through the opposite face of the collapsible plastic (ii) the dimensions of the Tuta bag were more acceptable in

the standard sized cup of the M.S.E. centrifuges (iii) the Tuta ports had a tamper proof seal mechanically better than the Dutch seal over the giving port entry (iv) the Tuta inflow lines were each separately controlled by a rigid plug which required pressure for its release before establishing the flow. These Tuta recovery bags provided an excellent system although one source of trouble was the fragmented plugs left in the bag during processing. When the angular standard plasma expressor was used for making the transfer of supernatant (Fenwal) the fragmented plugs sometimes became jammed in the jaws of the expressor with spicules of plastic perforating the bag face from within. A method of plasma and supernatant fluid expression had been developed by Mr. Muir using a rigid box assembly and lateral compression (not angular compression) to the bags such the contents (supernatant) could be expressed without danger of bag perforation. This obviated the problem of pin-hole leaks in the bags during processing. During this phase of the work numerous units of deplasmatised red cells were frozen in the system and recovered after varying intervals of time. Throughout these experiments observations were made on recovery of cells including:

1. Total red cell recovery as a percentage of the original frozen cell mass.
2. Supernatant haemoglobin estimation throughout the process as an indication of freeze-thaw injury.
3. Bacteriological examination of the blood during processing and especially at intervals during the shelf life of the recovered cells as one would

expect for a product which might be stored for at least 24 hours after recovery.

4. Haematological examination of the blood before and after processing to establish the changes, if any, occurring at this time.

5. Biochemical examination of the blood before and after freezing and thawing to assess the changes in electrolyte content of a product used for patient therapy. More sophisticated biochemical analysis of some more important assays concerning red cell metabolism were attempted including adenosine triphosphate levels before and after freeze-thaw and 2,3, Diphosphoglycerate levels both of which assays are known to be of considerable interest and importance in red cell viability and function (Mitchell, review 1976).

At this time consideration was given to the possibility of doing intracellular electrolyte levels, oxygen dissociation curves and in vivo red cell survival studies. These only highlighted some of the difficulties in working in a transfusion centre. Despite numerous attempts at enlisting the help of colleagues in academic departments it was not possible to pursue all of these problems. Recently we have made our own attempts but clearly expertise is needed as well as time and resources. Despite the work of Valtis and Kennedy (1954) on oxygen dissociation curves of stored blood there is no one in or around Glasgow capable of doing these studies (Professor Kennedy is now Professor of Renal Diseases at Glasgow Royal Infirmary). Throughout the period of this study numerous attempts have been

made to elicit the clinical response to the transfused frozen thawed cells. Unfortunately most clinicians have taken the view that anything put out by the Transfusion Centre must be good and they have nor or could not give any feed back of detailed information especially on oxygen requirements of patients before and after infusion and even such simple measurements as haemoglobin levels before and after infusion. Such information as did reach us in a haphazard way indicated that (a) the material supplied was therapeutic and without adverse effect (b) was acceptable to the ward staff regarding ease or administration (c) caused a rise in haemoglobin in the patients up to approximately 1 gram/unit transfused (d) cells survived normally as measured by a modified Ashby differential agglutination technique and a Chromium labelling technique (see Part VII) albeit on a small number of patients.

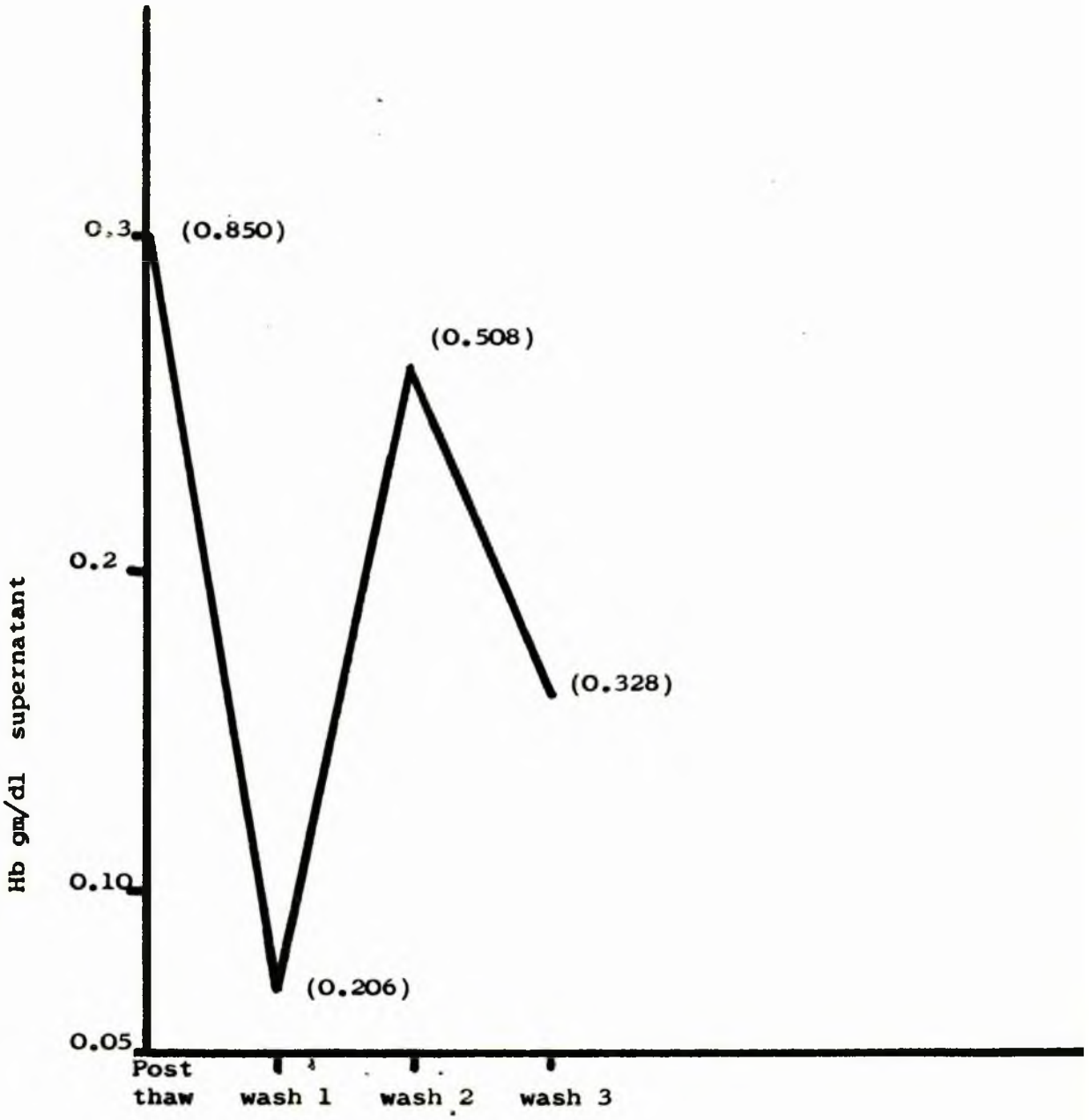
Such therapeutic acceptance as was indicated gave us sufficient confidence to proceed to phase 2 of the project. The first ever infusion of frozen blood was of course, a memorable day for us and came after we had established the techniques to enable us to prepare and transport frozen thawed cells in a sterile stable condition, now to be described in Part II.

Haematological Data

Haemoglobin concentrations of the deplasmatised donor red cells were performed before addition of glycerol, after glycerolisation, before freezing and after thawing. To assess the amount of cells destroyed in the processing - freezing, thawing, losses due to osmotically unstable cells, senescent cells and losses in the transfer tubing, supernatant haemoglobin levels in the immediate post thaw supernate and in the wash apparatus were performed. The summary of data (Table 2/2) is shown in the Table and is discussed in detail on page156. From these it can be seen that recoveries of 96% can be achieved. It is also possible to show graphically the so-called efficiency of process by showing the supernatant haemoglobin estimations as a function of time. In a good recovery the graph (fig. 2/3) should be of the type shown in the figure. The initial supernatant haemoglobin is high and rapidly diminishes after the first wash, during which some osmotic equilisation and cell squeezing occurs. During the second wash with normal saline there is an element of osmotic shock and some additional cells release haemoglobin which is then removed in the supernate. During the third wash a further lesser osmotic lysis occurs and on removal of the supernate one is left with a relatively stable product whose supernatant haemoglobin level should not exceed 200 mgm/100 ml. Since most of the third wash is in fact removed then when a small volume (70 ml) of normal saline is added as diluent the residual supernatant haemoglobin should fall well below the usually quoted acceptable level

	ACD	GLY	Final	No. of units examined ACD	GLY	Final
Hb gm/dl	11.72 ±2.35	12.92 ±1.49	17.62 ±2.95	121	120	116
PCV (v/dl)	38.64 ±6.77	42.58 ±6.6	57.64 ±7.84	91	90	112
MCHC g/dl	29.56 ±3.28	29.94 ±2.51	30.93 ±2.48	85	84	110
WBC/cubmm	4,447 ±1562	3,238 1,399	333 225	110	110	99

Table 2/2 Haematological values during process



Average Efficiency of Recovery
Figures in brackets show average losses of haemoglobin in grams at each phase of the recovery

Fig 2/3 & 4/3.

of 200 mgm/100 ml. Any residual free haemoglobin can be dealt with by the recipients haptoglobin binding mechanism for effective removal. Another index of therapeutic effectiveness has been used by various workers to assess the in vitro stability of the recovered red cells suspended in saline for 1 hour at 37°C. This test is meant to be a crude measure of the post thaw survivability of these cells but of course being an in vitro test is not necessarily indicative of the in vivo survival. There is no doubt however that the cells when suspended in saline at 37°C do show additional lysis and underline the need for their infusion into patients as soon as possible after recovery. To try to answer these and other questions some recovered cells were examined in two ways. (a) by the standard osmotic fragility curve and (b) by the measurement of in vitro haemoglobin supernatant build up over a period of days. These results are discussed on page 182.

Post Transfusion Survival

It did not prove easy to obtain the assistance of clinicians in deciding how the recovered cells behaved in vivo. This was probably partly due to two things. (a) we were working 22 miles away from the hospitals and so supervision of the transfusion therapy was not possible and inevitably specimens were not taken at the correct time sequence (b) the clinicians knew that frozen cells were therapeutic. They therefore assumed that if we approved then the cells must be clinically efficacious and that the response of the patients must be the

rain solution on the assumption that "the proof of the pudding is in the eating". This was not an easy assumption to resist and indeed we were anxious not to cause any undue alarm or throw any suspicion or suggest any doubts about the use of frozen cells. Nevertheless we were anxious to establish some facts and some useful data were provided by haematologists in the major teaching hospitals. All clinical data are analysed on page 134. It might be appropriate at this stage to indicate the manner in which a decision was taken to try frozen cells on the very first two patients to receive frozen cells from the first frozen cell bank in Scotland. This will be referred to in detail under clinical experiences but one can briefly state that patients felt better. They did not have any reactions, they did not excrete any abnormal pigment and no abnormal pigment appeared in their plasma. Their haemoglobin levels rose and were maintained above pre-infusion levels. Resident medical and nursing staff reported no difficulty whatever in administering the recovered cells. One consultant haematologist produced photomicrographs of a patient's peripheral blood film before and after infusion of red cells and two performed red cell survival studies using radiochromium labelling and Ashby differential agglutination. (see Part IV)

Biochemical Studies

In the early work or phase I it was possible to perform a number of basic biochemical analyses. With the assistance of Dr. Adam Flech, Consultant Biochemist at [illegible] Royal Infirmary electrolytes of blood before

and after freezing and after wash recovery were studied. These are described in detail, in Part IV.

In this way it was possible to show that electrolytes in the final supernatant diluent were for all practical purposes acceptable and especially that the potassium levels were especially low - a fact of considerable importance so far as transfusion was concerned in dialysis patients. Initially there was some resistance from local Biochemists to carry out these estimations which we had no means of performing ourselves due to lack of equipment but when the project was explained to Dr. Fleck he willingly agreed to accept a limited amount for inclusion in his routine hospital electrolyte measurements.

This meant a considerable saving in that an application had been granted for a flame photometer costing £8,500. His timely assistance meant that this expense was not incurred.

We were able to perform routine pH hydrogen ion determination on the various cell suspensions before and after freezing and Dr. Fleck corroborated these. These showed that the pH of the final product (washed recovered cells ready for transfusion) was slightly acid but rapid equilibration could be expected in vivo. Perhaps a more evenly buffered solute diluent would be more acceptable for resuspension of the cells. Such a fluid has recently been advocated by Valeri (1975).

Detailed biochemical studies on patients receiving frozen cells were not performed but again there was no adverse effect reported by clinicians. In a few 24 hour

post transfusion sera were examined for abnormal pigments by spectrophotometer and free haemoglobin measured by Porter's technique (1962) and Cripp's method (1965).

Since it is known that red cell viability in vitro and vivo is associated with levels of red cells adenosine triphosphate (Mitchell 1976) and since gaseous exchange is dependent among other things on the cellular level of 2,3 Diphosphoglycerate, (2,3 DPG) attempts were made to measure these on cells before and after freezing using commercially available kits for assay. (Sigma) The results to be described in detail are similar to those obtained by Blagdon (1973) and demonstrated that the levels of these substances are retained during the storage and immediately on recovery for up to 26 hours. This was very encouraging and strengthened the argument that cells should be laid down "fresh", - not more than 5 days from donation and these could then be recovered at any time in as good a state as when they were first frozen and placed in the frozen cell bank. This obviously is of importance in the physiological effect of such cells and presents the cells to the patient in as physiological a condition as fresh blood (Mitchell 1976).

Trace Metal Studies

Since Shaw had considered that aluminium cannisters for the long term preservation of frozen cells might present a hazard to patients by slow accumulation of aluminium in the frozen cells during storage in the systems used it was important to try and assess the levels of aluminium likely to be encountered (Shaw 1972).

There were two possible ways of investigating this. Firstly using a modified flame photometric system and emission spectrometry. In discussion with biochemists it was found that aluminium was not an easy element to analyse in this way because of interference by other elements. Also of course, we did not possess the necessary equipment nor could we persuade a biochemist to provide it. The second approach concerned the system of activation analysis using a high energizing source of atomic particles to convert aluminium into a radioactive phosphate radicle and its subsequent isolation and radiochemical analysis. I had seen such studies on total body iron, B12 and electrolytes being reported at the local Glasgow Blood Club in collaboration with Dr. Body of the National Engineering Laboratories' radioactive reactor at East Kilbride's Scottish University's Centre and after consultation with Dr. Body and Dr. East a method was established to measure the quantities of aluminium in normal donors and blood before and after freezing following storage for various intervals in the liquid nitrogen system. These studies proved peculiarly difficult but some figures were obtained to show that even after prolonged storage only infinitesimal quantities of aluminium could be found in the frozen cells during thawing and especially after the washing was complete. The normal level of aluminium in donors is very low and there would not appear to be any appreciable gain in aluminium in the final product. Shaw had of course, commented on the problems of excessive amounts of aluminium which could seek bone and bone forming tissue especially in renal dialysis and uraemic patients

showing evidence of demineralization of bone due to secondary or tertiary hyperparathyroidism. Such circumstances might exist where patients (uraemic) consumed large quantities of aluminium hydroxide in the treatment of associated peptic ulcer. Shaw considered that aluminium might be a bone-seeking element which exchanged or replaced phosphate ions.

No adverse effects of such long term ingestion of aluminium salts on bone metabolism have ever been demonstrated compared with the well known toxic effects of excessive amounts of other metals such as zinc and cadmium but Shaw was concerned lest there should be some long term more subtle effect with a long latent interval. The point was also made, of course, that aluminium cooking vessels probably add aluminium to the dietary intake. All of this begs the question however since any aluminium in the frozen cell product should be delivered intravenously which might have an effect if present in any substantial quantity. These experimental results are reported in part IV.

In fact the amounts of aluminium found are very small and of the same order as normally found in human plasma. No studies have ever been done on aluminium metabolism in man and no attempts were made to detect aluminium in the urine of patients receiving frozen cells because it was almost certain that the infinitesimal quantities diluted in the urine would be beyond the limit of detection.

Intermediate samples during the freezing and thawing were examined as well as the wash solutions to determine

that there would be no net gain of aluminium from sources other than the cannisters. Since there is a four-log wash at the recovery phase then this in itself would be expected to reduce the aluminium levels by simple dilution and removal in the supernatant. It could be argued however that aluminium leaching from the can during storage was sequestered in two hypothetical mechanisms. (a) adsorbed onto the suface of cells or (b) carried intracellularly. All that one can say in answer to these suggestions is that the chemical methods of releasing the aluminium from the system (see Appendix) would as well as disrupting the cells have been expected to release the cell contents for measurement. No such release was detected. After thawing it can be seen that the inside of the aluminium can does show evidence of spots of discolouration such as one would see in a corrosive process. Electrolytes are present in the frozen blood both in the autologous residual plasma and in the glycerol protective solution so that one might expect some electrolytic dissolution by electrolysis. At temperatures below 130°C when all the water is turned to ice and no further phase transition occurs in the system it is doubtful if much ionic exchange could occur. Of course, it is possible but the author is not an expert in cryogenic engineering to answer these points. Colloids are known to prevent electrolysis in such circumstances and this is likely to occur in a glycerol - plasma medium. One must however agree that the corrosion can be observed in small discrete spots and for this reason the aluminium cans are not reused for fear of weaknesses in the wall.

Before leaving the question of aluminium it is as well to record that after these studies were begun I learned of similar findings in the Brentwood studies, and later still in Edinburgh studies, where although flame photometric methods had been used by their collaborators, small barely detectable amounts of aluminium were the rule. (Blagdon 1973; Pepper 1975).

Bacteriological Studies

Since multiple transfer manipulations of any blood product result in multiple opportunities for infection a close study was made of the possible contamination of the washed cells by bacteria. Direct films of the blood before and after freezing were made and as well as growth plates on nutrient agar and blood agar at room temperature and 37°C, incubation was continued for 7 days. Liquid cultures of 10 ml blood before and after freezing and recovery of the final product were made in Brewer's yeast and Robertson's meat broth media for 7 days at room temperature at 37°C. Facilities exist in our Centre for the subculture and identification of any organism. Careful transfer techniques and laminer flow work cabinets are used for the multiple transfers. In no case was there any contaminant introduced into the product at the time of wash recovery. Occasional gram positive cocci (staph albus) were to be found as "sampling errors" in subculture at the end of prolonged incubation. At no time was there any irruptive growth of pathogens even when cells were kept at 4°C as a saline suspension for up to 14 days. This will be described in detail.

It is important to record that all of these cryo-protective and wash solutions are made in our own premises

to our own formulation. (see Tech Appendix). This was not so surprising as may first appear since the West of Scotland Blood Transfusion Centre had had the responsibility of manufacturing sterile water for parenteral intravenous use for reconstituting dried plasma for many years. There was therefore adequate technical resources to achieve this as well as the associated bacteriological surveillance to maintain high standards of sterility up to B.P. requirements. These stands could also be tested for pyrogens by the standard rabbit method similar to that used for testing water and blood products. (B.P.1973).

Despite the encouraging negative bacteriology findings in all of the units tested it was decided that even as we became more and more confident of the ability to recover cells aseptically and maintain sterility this was so important that it was decided that whatever other tests were abandoned we must not stop testing the final suspension of cells prior to infusion. That rule still applies today after infusion of thousands of units of recovered cells. Reference to the second ever patient to be transfused will illustrate this point. The patient a 50 year female had had multiple transfusions of conventional blood because of aplastic anaemia and developed multispecific leucocyte antibodies so much that every time she received even washed concentrated red cells she had unpleasant allergic reactions which made her complain bitterly each time. As time progressed her reactions became more severe until she dreaded the thought of any further

infusions. She agreed to try frozen blood in an effort to minimize the leucocyte load and arrangements were made for her to receive frozen cells on her next requirement. When the day arrived and just as the washed frozen cells were about to be despatched to her the consultant in charge telephoned to say that she had developed a high pyrexia which he thought might be due to septicaemia! It was subsequently shown that she had indeed developed a pyocyaneus septicaemia which although it finally responded to antibodies took some time to resolve. One wonders what would have happened if the frozen cells had been infused on the day previous to the first spike of temperature. One might have been justified in thinking that the frozen cells had something to do with it and we might have abandoned all further work at that stage! Perhaps Divine Providence had taken a hand in deciding the future of frozen cells. This patient, to complete the story, eventually got frozen cells on numerous occasions and each time she commented on the lack of reaction and expressed her thanks. She lived a further 2½ years punctuated by regular visits to hospital and ultimately died of an overwhelming respiratory infection.

Perrault et al (1967) described a similar case of a 38 year old leukaemic woman who had had two units of conventional ACD blood every week for 12 months. Each transfusion was accompanied by marked pyrexial and pyrogenic reactions despite buffy coat removal and saline washing of the ACD blood. Frozen cells (15 units over 2 months) were given without any reactions and

haematocrit was maintained. Another similar 58 year old woman observed that after frozen cell administration she "did not feel warm" as she had done with ACD blood and that she much preferred the frozen blood.

Huggins (1963) recorded that removal of white cells and platelets during processing for patients on whom febrile reactions occur during conventional transfusion made patients "very grateful".

When pyrogen testing was done with the supernatant from the last wash using previously non-pyrogenic physiological saline, no pyrogenic reaction was encountered. This was predicted but the tests were carried out in the early stages of the work to assess if the unexpected might happen. Thus although the aluminium cans were chemically cleaned, neutralised, rinsed and cleaned with pyrogen free water (see Appendix) some felt that pyrogenic material might leech form the can walls during freezing-thawing and during cryogenic storage. Some were concerned that the original glycerol might be pyrogenic or that the original sorbitol wash solution might be pyrogenic. I argued that each of these substances were removed during the wash phase and that testing the final supernatant recovered from the cells before transfusion would answer the questions. This proved to be correct and no instance of pyrogenic material was found. (see letter from Appendix). Since pyrogens can adsorb onto activated charcoal it was possible that adsorption on to the red cells might occur and this would free the supernatant of pyrogen. The only way to answer this was (a) by infusion into rabbits and (b) infusion

into humans.

Accordingly three rabbits were each given 20 ml frozen recovered cells without any effect whatsoever and without any pyrogen response. This experiment also served the dual purpose of making some anti-M and anti-N in rabbits since MN red cells were used. Later as clinical acceptability was built up on the use of frozen cells in selected cases more and more it became obvious that in humans there were no pyrogenic responses and no untoward effects.

Clinical Indications for the use of Frozen Cells and need for a Frozen Cell Bank

The clinical indications for the use of frozen cells are numerous (see Table 2/4). I propose to take each of these in turn and to assess the position concerning each claim. At the same time it is only fair to assess the claim to the contrary and the case against the use of frozen blood.

Availability of rare groups. There is no doubt that some donor cells are rare in their red cell antigen make up. Such donors may be used for patients who had alloantibodies to these rarer antigens but clearly two things change which make the task of finding a donor difficult. (a) the degree of difficulty is directly proportional to the rarity of the antibody and scarcity of the donor. (b) the scarcity of a suitable donor may reflect his innate scarcity and the fact that he may not be readily available when required because he is not at home, is unaware of the problem, is medically temporarily or permanently unfit to donate or has donated within the past few months. In such circumstances as

FOR OR AGAINST FROZEN CELLS

Against

Increased costs

Increased process time

Short shelf life

Recipient hazard - contamination in immunosuppression

? Removal of viruses, leucocytes, platelets

For

Availability of rare groups

Physiologically superior product

Reduced risk of hepatitis

Reduced risk of alloimmunisation to tissue antigens

Reduced incidence of febrile reactions

Facilitates component therapy

Solves inventory problem

Permits autotransfusion

Booster immunisation programmes

Military requirements

Table 2/4.

these it is possible to maintain a local, national or international panel of rare donors who can be contacted usually for a planned procedure and rarely for an emergency. Since only a few donors may be able to donate for a particular patient there is no means of obtaining any more once these donors have been bled and, of course, it may not be possible to obtain a donation for the reasons outlined. What then is one to do in these circumstances? A system of long term preservation of donations allows accumulation of stocks of rare blood which is independent of later illness in the donor or non-availability of the donor. A record can be kept in the same way as the national and international rare donor panels provided samples can be obtained and stored with the frozen cells matching tests to the patient's serum can be performed before the blood is thawed out and released. In this way it is possible to accumulate sufficient blood to perform a procedure on a patient or, of course, as a corollary on the donor! Against these advantages of frozen cell banks are the objections that a frozen cell bank is of no value if the patient is some distance away (perhaps in another country) and that if the frozen cells are sent in the frozen state the receiving laboratory (perhaps in a technologically less well developed country) must be able to do the recovery. These two arguments are not valid however since other rare banks also have transport problems and even if a frozen cell recovery programme is not available at the receiving laboratory it is possible with proper regard to transport schedules to send viable recovered cells already recovered.

The best plan is for the receiving laboratory to have a method of recovery available and staff to operate this. Only in this way can cells be recovered if and when they are needed. It would be tragic to recover cells, send them to a distant patient only to hear that the operation had been cancelled or the patient did not, after all, need transfusion. These arguments strengthen the argument among "blood freezers" in U.K. that (a) we must maintain uniform standards of freezing and recovery and (b) we must plan for long distance transport of frozen cells. As far as uniform standards are concerned, in the early 1970's there were only three laboratories in U.K. capable of freezing and recovering cells: Army Blood Supply Depot, Brentwood and Glasgow. Reference has already been made to the close co-operation between these departments and from this there emerged a uniform methodology which has stood the test of time and been introduced by all but one of the laboratories taken up frozen cell banking - Guy's Hospital, Bristol, R.T.C. Aberdeen R.T.C., Inverness R.T.C. and Leeds R.T.C. More recently other centres have used other methods and some are contemplating introducing entirely different systems (Pepper 1976).

Whilst we have no desire to rigidly dictate which method should be used and recognizing the need for improvements in our system it seems that these workers will only be content to pursue methods which will inevitably mean delays due to a need to do the basic research and feasibility trials in U.K. and which on the evidence available, are vastly more expensive at a

time when national resources are not necessarily available. Methods may be introduced which cannot be compared with existing methods. Technicians will be trained to work one system but not the others and so on; the whole object of interchangeability and transport of frozen cells from one centre to another will become impracticable. To investigate the feasibility of two systems of transport we have performed three different types of exercise to be reported in part III.

Early in phase 1 in collaboration with Drs. J.W. Jenkins and J. Blagdon of the Regional Blood Transfusion Centre, Brentwood, Essex an exercise was planned in which frozen cells would be sent by road from Brentwood to London Heathrow Airport for shipment by commercial airliner to Glasgow Abbottsinch Airport where the cells would be collected and delivered to the Regional Blood Transfusion Centre at Law Hospital, Carluke. The transport arrangements were made by Securicor Services and four cans of blood stored in a standard British oxygen company small dewar of the ODS, type were placed over blocks of absorbent material previously filled with nitrogen in the bottom of the flask. In this way no actual liquid nitrogen was free to splash about. The cans were held upright by cardboard spacers and the lid ventilated to allow escape of vapour during the journey. One telephone call from Brentwood to Law was made on the appointed day saying the frozen blood had been despatched and we should stand by to receive it. This presented no difficulties and no special instructions

were required until Securicor arrived with the consignment. Thereafter thawing and processing were by the standard procedure. Technical staff had no complaints and the four units were processed just as though they had come out of our own bank. One minor criticism was that, to save valuable stocks, Dr. Blagdon had in fact sent specially frozen time expired (21 days ACD) blood so that when deglycerolization was complete, supernatant haemoglobins were higher than one usually finds with blood from the frozen bank which is not more than 5 days old when frozen down for inclusion in the bank. All four units were sterile on extensive routine bacteriological testing and blood films of the recovered cells showed normal morphology.

There problems of transport are further studied in part IV.

PART III

RESULTS OF STUDIES ON PROBLEMS REFERRED TO IN PART II

RESULTS AND REFLECTIONS ON STUDIES MADE

"The frost which kills the harvest of a year

Saves the harvest of a century, by destroying
the weevil or the locust".

Emerson, Conduct of life: consideration by
the way.

Metabolic Integrity of Red Cells after Cryopreservation in Glycerol

The major portion of the energy reserve in the mammalian red blood cell is represented by its content of ATP (Mitchell 1976). Among the relationships which are closely correlated with this index are the active transport of sodium and potassium, the viability of the cells (Jones et al 1957; Nakao et al 1962) following transfusion as measured by survival and resistance to haemolysis, the maintenance of shape and the ability to reinitiate glycolysis (Tullis and Lionetti 1966). In contrast to the ATP depletion that occurs during conventional storage at 4^{o}C (Mitchell 1976; Bartlett and Barnett 1960) cryopreservation by the low glycerol rapid freeze method leaves the energy status of the red cells unimpaired. ATP concentrations are adequate for the resumption of the various ATP dependent cell functions after cryopreservation as they were before freezing. Frozen thaw recovered cells lose their activity at the same rate as non frozen cells. Intracellular potassium concentrations are unaffected by the low glycerol and rapid freeze technique of cryopreservation and recovery in sharp contrast to the

depletion which occurs in conventional liquid storage and in high glycerol -80°C method (see p.68). Permeability of the erythrocyte membrane before, during or after freezing and thawing is unaffected and behaves as well as in the conventional system of banking blood. There is however a slight gain in intracellular sodium into resuspended cryopreserved red cells. Incubation at 37°C quickly restores any inbalance and the cryoprocessed cells transport sodium and potassium equally as well as non-processed cells.

Valeri et al (1970) also pointed out the importance of preserving red cell function as well as their physical integrity and red cell survival. The relationship between red cell 2,3 Diphosphoglycerate (DPG) and adenosine triphosphate (ATP) and oxygen carrying power of red cells has been stressed by numerous authors (Mitchell review 1976). Since glycerol freeze preservation procedures maintain 2,3 DPG and ATP along with normal oxygen uptake and oxyhaemoglobin dissociations when red cells are frozen within 24 hours of collection they offer advantages if transfused within 24 hours of their recovery so far as retaining oxygen transport function and 2,3 DPG (O'Brien and Watkins 1960; Valeri 1974; Derrick et al 1972; McConn and Derrick 1972). Sartian, Rowe and Gottfried (1971) showed that low glycerol rapid freezing with storage in liquid nitrogen preserved the total lipid, cholesterol and phospholipid as well as recently donated fresh whole blood. This further strengthens the suggestion that with the low glycerol

rapid freeze method at least the integrity of the cells is preserved as well as that of non frozen non processed erythrocytes. Almond and Valeri (1967) however had shown previously that there was no correlation between survival or length of post thaw storage in autologous plasma up to 11 days at 4°C with any red cell lipid fraction. Krijnen et al (1964) using cells recovered from a low glycerol liquid nitrogen bank showed that metabolic properties, osmotic fragility and post transfusion survival were satisfactory with cells showing normal or near normal glycolytic capacity, ATP levels, and plasma potassium concentration.

The major energy producing compound in human erythrocytes is the nucleotide adenosine triphosphate (ATP) the content of which depends on the glycolytic capacity of the cells (Mitchell 1976). The maintenance of osmotic resistance to haemolysis (Nakao et al 1960), the preservation of shape (Nakao et al 1961) and maintenance of ionic gradients between the cells and plasma are dependent on adequate ATP concentration (Hoffman, 1962). A positive correlation between the ATP content of liquid stored erythrocytes and post transfusion survival has been observed (Gabrio et al 1956; Jones et al 1957; Nakao et al 1967) and considerable emphasis has been placed on defining the composition of media used in erythrocyte preservation in order to maintain adequate nucleotide synthesis (Bishop 1964; Gabrio et al 1955; See Mitchell 1976 for full discussion and references). Lionetti et al (1966) showed that recovery of cells by cytoagglomeration using Huggins method resulted in low recoveries of ATP in resuspended processed cells whereas

centrifugal washing type recovery resulted in unimpaired recovery. Prolonged contact with hypertonic glucose resulted in rapid crenation and deterioration on return to isotonic non-buffered media, whilst resuspension in autologous plasma restored some of the lost activity. Post thaw storage at 4°C resulted in gradual loss with rapid deterioration from 8-21 days. Valeri et al (1966) found no correlation between in vivo survival and ATP content of recovered cells from Huggins method. Correlations were noted between the in vivo survival and hexokinase, glucose 6-phosphate dehydrogenase, glutathione reductase and glutathione stability. Their view was that the selected method preferentially preserved young cells rich in these enzymes and substrates. Deleterious effects on the cells were reflected in increased density and changes of shape in the recovered cells.

Akerblom and Hegman (1974) using low glycerol rapid freeze to -196°C in a Teflon-FEP Laminate freezing bag reported that the recovered red cells were equivalent to those of fresh ACD blood in respect of ATP, 2,3 DPG and post transfusion survival. Post thaw storage in saline glucose resulted in a progressively increasing supernatant haemoglobin and addition of ACD or ACD adenine plasma (compatible or autologous) prevented this. A small amount of CPD solution also kept the supernatant within acceptable levels for at least 24 hours. A later paper by these same authors dealt with recovery using a continuous flow counter current washing system (Fenwal Ultra flow Elutramatic system 83b) but although red cell in vivo viability

was good, in vitro recovery was poor, ATP and DPG levels were maintained at pre-freezing levels and intracellular potassium was reduced by 20%.

Metabolic integrity of cells preserved by Extracellular Protective

Kinfret et al (1965) in evaluating the various techniques, which they introduced with the Linde Company rapid freeze apparatus found that cells recovered from PVP/Dextran could be kept at 4°C for 1 week as a saline suspension with added glucose with no more than a loss of 2.5% of the cells. Results were also modified depending on whether the protectant was added to whole blood in ACD, partially packed cells in ACD or anticoagulant protective mixtures at the time of venesection. They described a "saline stability test" which has been used occasionally since in order to assess the post-thaw stability of cells when suspended in normal physiological saline for 30 minutes (Robson 1970). Unfortunately this test has been interpreted by various workers in various ways. Some take the method to mean a 1 in 50 dilution of the final red cells immediately after thawing and 30 minutes after resuspension of an approximately 2% haematocrit solution at room temperature (Robson 1970). Others have assessed stability as meaning resuspension in 40 to 100 volumes of physiological saline (Doebbler et al 1966; Kinfret et al 1965). Strumia and Strumia (1965) described 3 forms of stability test after thawing:-

(1) recovered cells remain undisturbed in their supernate

(2) resuspended cells in autologous plasma in proportion 1:9

(3) resuspended cells in saline solution in proportion 1:9

The high dilution was chosen to stimulate the dilution effect of a transfusion in a 75 kg man. No relationship was found between these tests and post transfusion survival. Oxygen dissociation curves on the recovered cells showed that they were very inferior to fresh cells and equal to 20 day old ACD banked blood. Yet others concerned with the use of glycerol have used incubation of cells at 37°C in a physiological saline medium for up to 12 hours (Rowe et al 1970) during which many more measurements are made on the metabolic and functional integrity of the cells and not just supernatant haemoglobins. Nevertheless the test is of value in deciding on the likely survival of recovered cells since gross haemolysis would indicate a very unstable cell which would be useless therapeutically and might be dangerous if given intravenously. It is therefore no more than a useful screening test whose exact technical description has never been adequately laid down since it means different things to different people (Gikas et al 1965). Robson made some use of this test however in assessing the effect of various colloids in red cell preservation - PVP, Dextrans, Hydroxyethylstarch. (Robson 1964, 1970).

Post transfusion survival studies on the PVP method of Kinfret et al were reported by Bloom et al (1965). Optimal survivals were 88 ± 6%, 24 hours after infusion. Strumia and Strumia reported 69% survival for cells

protected with Dextran 40 and 62% for cells protected with albumin. As well as these survival studies they reported supernatant potassium levels of 14.9 mg/l ± 1.8 free haemoglobin 9.6 gm/100 ml ± 0.40 - an exceptionally high figure when dextran 40 was used, and 19.8 mg/l ± 0.61 potassium, 10.4 gm/100 ml ± 0.43 supernatant free haemoglobin when albumin was used. These results are so disappointing when compared to the rapid freeze low glycerol methods that it is hardly any wonder that they have not enjoyed must popularity in Civilian practice. Furthermore oxygen dissociation curves showed that the recovered cells although fresh when originally frozen behaved more like 20 day old banked blood with a distinct shift to the left (Mitchell 1976).

In work with PVP Gikas et al (1965) showed that again there was good recovery of cells with PVP mol. wt. 25,000 or 40,000 but supernatant potassium levels were excessive as were haemoglobin levels in the supernatant - all indicating some post thaw damage confirmed by demonstration of the abnormal post thaw saline suspension stability with "recovery" dropping to 86% and in vivo survival at 24 hours of 27% to 81.3% signifying that some in vivo haemolysis was occurring sufficient to overwhelm the haptoglobin binding mechanism and the renal threshold for free haemoglobin in all cases. Although lower molecular weight homologues of PVP gave equal protection and would be excreted from the body in the urine without long term storage in organs, a cautionary note was introduced when it is realised that small molecular weight colloids, of course, do have an

effect on expanding the blood volume which could seriously embarrass the circulation in cases of compensated anaemia or cardiac insufficiency. (Ravin, 1952).

Vinograd-Finkel et al (1968) reported to the 12th Congress of the International Society of Blood Transfusion in Moscow about their work at the Central Institute for Haematology and Blood Transfusion Moscow. PVP gave recoveries in vitro of greater than 94% and free haemoglobin was 70 ± 20 mgm/100 ml to 195 ± 41 mgm/100 ml. Cells stored at 4°C for up to 5 days after recovery from freezing showed only a 1% rise in haemolysis. They considered that it would be safe to transfuse 1 unit of PVP frozen blood but did not actually do so - preferring to infuse animals and restrict human use at that time. The in vivo survival of small 10-20 ml aliquots of autologous PVP preserved cells showed acceptable acute post transfusion survival but in vitro losses ranged from 3-4% with supernatant haemoglobins of 1500-2000 mgm when full units were tested. These also showed supernatant potassium levels of 16 meq/l and 35 gm of PVP (almost the limit) as well as red cell debris (Whitcher 1964). The latter author reported that in 5 selected patients who each received 2 units of PVP preserved blood, 4 developed haemoglobinuria with no evidence of acute renal insufficiency. Valeri (1966) cites Richards et al (1965) that in vitro survival of PVP preserved cells was 82% at 24 hours, higher than expected plasma haemoglobin levels were found as well as a supernatant haemoglobin of about 2.8 gm/unit of cells. Of 10 patients seven had transient haemoglobinuria after two units of blood which also contained 70 gm PVP

which could be detected readily in the patient's plasma, in one case for as long as six months afterwards.

Altemeier et al (1954) showed that PVP can be retained in body organs and reticuloendothelial system with inflammatory or granuloma foreign body reaction. This resulted in a general slowing of interest in PVP and the National Research Council Panel on plasma in U.S.A. suggested that the total amount of retained PVP infused into a person in his lifetime be limited to about 47 gm. (Bernhard et al 1954).

Lavrik et al (also Russian) reported to the same meeting (1968) that low molecular weight PVP (12,600) at 10% concentration gave recoveries of 95.3% with haemolysis of 4.7 gm $\pm$ 0.28/100 ml and potassium levels of 42.6 $\pm$ 1 mgm/100 ml. Post transfusion survival at 24 hours was 82% ($T\frac{1}{2}$ = 14 days) but these results were established in patients with malignant disease. Unfortunately no actual numbers of patients are quoted nor is there any indication of the types of malignant disease and if these could account for such low in vivo survival values.

de Verdier et al (1965) investigated the cryoprotective properties of Dextran 150, (Molecular weight 150,000) in samples of blood stored in liquid nitrogen in small flat bags made of 0.05 mm thick aluminium foil (total capacity 12 ml) in thin plate formers 0.5 cm thick. Dextran or Albumin could prevent both freeze thaw haemolysis and post thaw processing haemolysis. The best protection was found with Dextran 40 (mol wt. 40,000) although even Dextran 20 was protective. Nevertheless in all of these formulations high levels of haemolysis occurred

especially during the washing phase in buffered saline.

Morrison and Mollison (1966) commented on the difficulties of performing radio chromium survival studies on PVP preserved red cells since the cells are labelled in the presence of the PVP and cannot be washed free of it before being reinfused.

Doebbler et al (1966) recorded recoveries of 96.9 ± 0.4% with a 30 minute saline stability recovery of 89.1 ± 1.5 for full pint donations of blood in ACD + 7% PVP using corrugated aluminium containers for freezing in liquid nitrogen. Resuspension of thawed cells in saline for longer than ½ hour showed an increasingly poorer recovery until at 168 hours later saline suspension recovery was 85.4%.

Robson (1970) described his experiences with extracellular cryoprotectives in the storage, by freezing in liquid nitrogen, of whole donor units of blood in Dextran. He commented on the generally poor recovery of cells over a great range of experimental conditions and it varying molecular weights. PVP had similarly been tried at 7.5% w/v with recoveries of 96.4 ± 0.05% but poor red cell survival in vivo of 73.5% at 24 hours. Hydroxyethyl starch was also tried. It is similar to Dextran and was originally developed like Dextran and PVP as a volume expander. It is considered non-toxic and non-allergenic (Ballinger et al 1966; [illegible]tub et al 1969; Metcalf et al 1970; Brickman and [illegible] 1966; Maurer and Berardinelli 1968). Baar (1972) reported on the use of hydroxyethyl starch as a cryoprotective following the early demonstration of this

property by Knorpp et al (1967). She combined the HES with albumin in varying concentrations and found the optimum, so far as in vitro testing was concerned, to be a combination of 14.7 gm salt free albumin/100 ml in ACD saline plus 11.7 gm/100 ml HES. Recovered cells were resuspended in autologous plasma. Robson (1970) reported on in vitro red cell recovery of 96.5 to 97.0% using 15% HES in ACD with much larger units of blood than Baar. Knorpp et al (1967) reported a 13% loss after freezing red cells in 14% HES in 0.9 gm/100 ml NaCl. Conflicting views exist on the effects of HES on platelet function and coagulation mechanisms although recently Weatherbee et al (1975) found no adverse effects when HES was given with homologous or autologous blood frozen in 14% HES to shocked monkeys. It is still not certain if HES will find a place in the search for the ideal cryoprotectant for use in military or mass casualty situations as a stable, non-toxic, bio degradable efficient cryoprotectant which does not require removal before infusion. Since these methods of non-penetrating additives are supposed to have the advantage of not requiring any post thaw processing it is important to remember that all previous workers have found that the temperature conditions are critical to good recoveries of cells and that most of the freezing, thawing and administering is done in one container. Unless a wash phase is introduced (thus effectively destroying the raison d'etre) one cannot see clearly the actual blood to be administered. Valeri suggested that plastic containers might give a better view of the contents but at that time (1966) no suitable cryos' ble plastic was available and I know of no such experiments with

the newer Teflon bags.

Simple centrifugation, of course, removes about 80% of the additive and supernatant haemoglobin but this requires transfer of the thawed cells to a suitable bag from which the cells can be transfused or washed further or both. With two serial centrifuge washings, the total PVP can be reduced to 0.1 gm/unit and supernatant haemoglobin to 40 mgm/100 ml with a 10% in vitro cell loss. Thus the whole purpose of the non-penetrating additive has been lost.

PART IV

EVALUATION AND PRACTICAL RESULTS OF FROZEN CELLS

"Blow, blow, ye winds, with heavier gust!
And freeze, thou bitter biting frost".

Robert Burns, A Winter's Night.

OPTIMAL CRITERIA OF ACCEPTABILITY

Valeri (1966) outlined the requirements of the American National Research Council Committee on Blood Transfusion Problems to establish the clinical acceptability of frozen cells. Pilot studies were done with 10 ml samples to define the optimum conditions at all stages of the process from the collection into anticoagulant, separation, pre-freeze storage conditions, concentration of additive necessary for optimum protection, storage time in the frozen state, optimum conditions for thawing and post thaw treatment to remove the products of haemolysis and additives, resuspension medium composition and resuspension storage at $4^{o}C$. Thereafter full unit homologous transfusions to stable anaemic recipients established post transfusion survival by the Ashby differential agglutination method using the Model B Coulter Electronic Particle Counter (Green dyke et al 1965) as well as measuring vital signs plasma and urine haemoglobin, serum bilirubin, potassium levels, central venous pressure and urinary output.

The United States National Research Council National Academy of Science Committee on Blood Transfusion Problems suggested guidelines to evaluate the results of various freeze preservation methods (Valeri 1969). They divided the uses of frozen blood into two major groups.

1. The limited requirements for special circumstances -

rare group, autologous transfusion, surgical procedures requiring large quantities of blood and

2. The circumstances in which ACD blood is usually given.

In group 1 the operational costs and efforts involved in providing this facility were not considered relevant since the frozen cell preparations was a unique biological product with certain defined advantages over ordinary banked blood e.g. no "ordinary" donor was available.

In group 2 the costs and effort involved were considered to depend on whether or not the frozen blood was to fulfil the entire demand or supplement a failing ACD bank.

Apart from these considerations of organisational and financial importance it was also important to ensure certain biological criteria equal to or greater than for ACD blood.

1. Total loss of RBC, 24 hours after transfusion must be acceptable (minimum 70%) and surviving cells should have a normal life span in vivo.
2. Frozen cells should not contain any toxic additive which cannot be excreted or rapidly metabolised or is likely to sensitise the patient by reason of any antigenic component.
3. Preservation should be possible for at least 1 year.
4. Physiologically and bacteriologically the recovered cells must behave as well as 21 days red ACD bank blood.
5. The immediate and long term conditions of the

recipient's health should not be prejudiced by any untoward or secondary effect from a single or multiple unit transfusion of preserved cells.

6. If possible the cells should be packaged to allow the use of the same container for collecting freezing, thawing and transfusing. This would ensure accurate donor identification and minimal risk of bacterial contamination.

The manner in which these various criteria have been met will be examined in this Part IV and results of our own studies evaluated.

EFFICIENCY OF RECOVERY

A consecutive series of 95 recoveries were studied in detail to investigate the efficiency of the recovery and washing process. The Table shows the supernatant haemoglobin in the various phases of washing process and these are depicted graphically in the figure on which are superimposed the mean and standard deviation figures for each stage of the process:

Table 4/1 Efficiency of Recovery

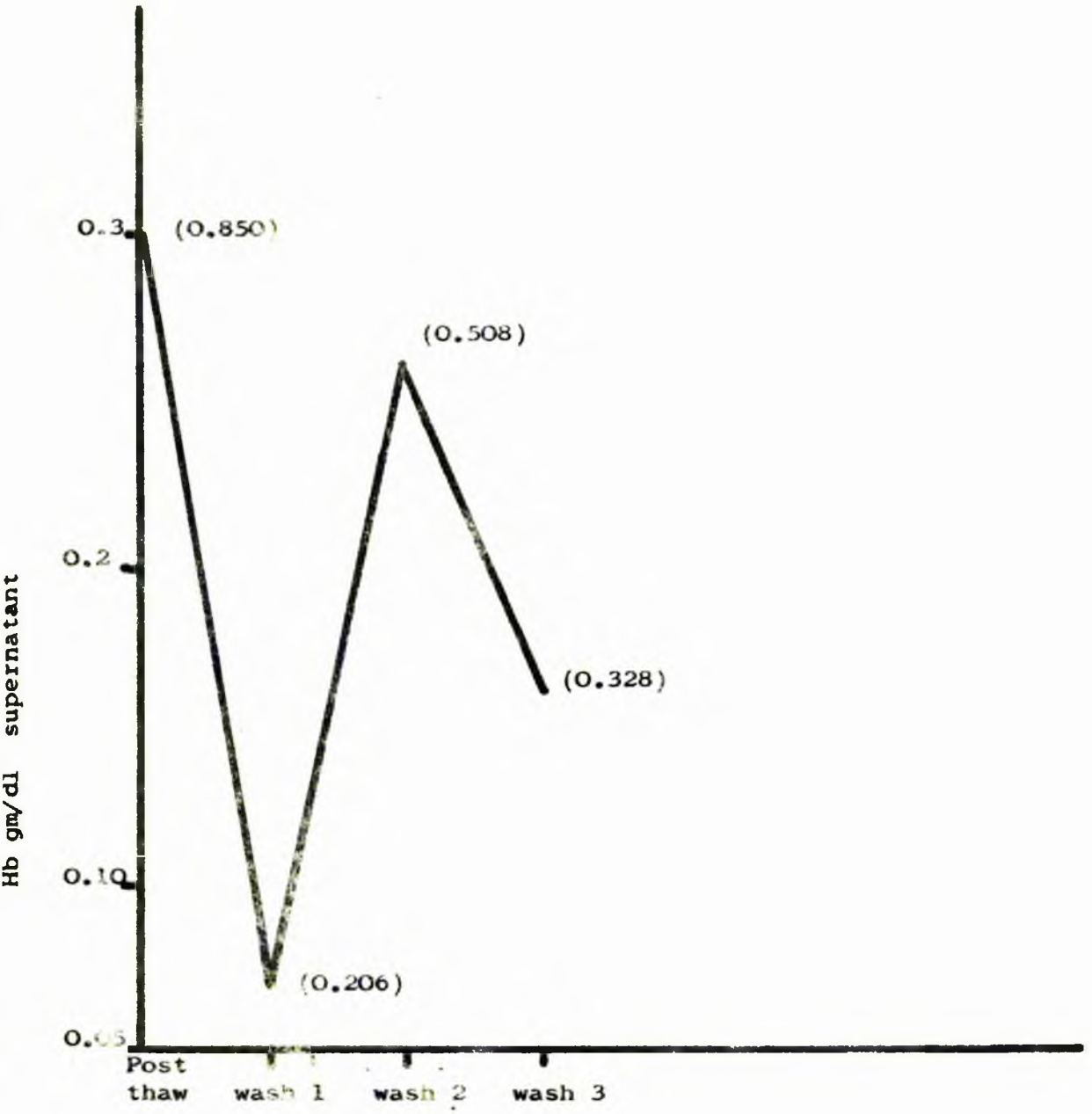
	Mean	± S.D. gm Hb/dl	
Post Thaw supernate	0.3133	0.1002	ν= 95
Sorbitol Wash 1	0.0671	0.0373	
NaCl Wash 2	0.2613	0.1466	
NaCl Wash 3	0.1604	0.0995	

Total losses of haemoglobin in the supernate were calculated from the volumes of supernatant removed and their haemoglobin concentrations. These are shown in the Table and superimposed on the Figure in order to illustrate the point at which the various losses occur.

Table 4/2 Total loss of Hb gm/dl

	Mean	± S.D.	ν = 95
Post Thaw Supernate	0.8499	0.4404	
Sorbitol Wash 1	0.2058	0.1289	
NaCl Wash 2	0.5077	0.2547	
NaCl Wash 3	0.5278	0.1781	

From these figures it can be seen that the efficiency of the processing is excellent and results in the production of a final supernatant wash of 160.4 mgm Hb/dl - well within the requirements already noted (see 117). It has

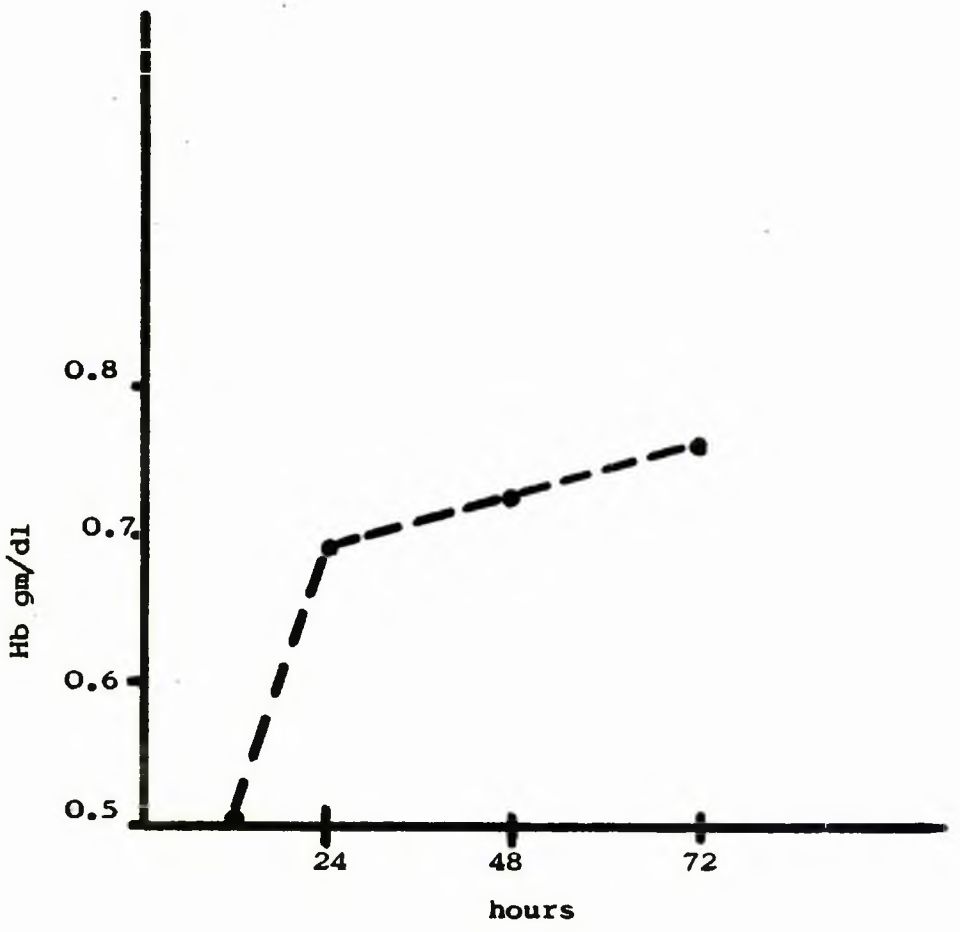


Average Efficiency of Recovery
Figures in brackets show average losses of haemoglobin in grams at each phase of the recovery

Fig. 4/3

also to be remembered that all of this total haemoglobin in the bag at the time of the last sodium chloride wash does not actually go into the patient since most of this is removed as final supernate (250 ml) before the addition of 70 ml of fresh NaCl to reduce the viscosity. Thus the residual haemoglobin actually given to the patient will be very much reduced of the order of being diluted approximately 1 in 100. (p 117).

Most of the losses in the recovery clearly occur as a result of direct freezing and thawing injury (Krijnen et al 1964). Although the removal of the initial glycerol haemoglobin supernate is followed by a rapid fall, on the addition of sorbitol wash some water gets into the cells and causes an indirect osmotic lysis which on removal causes the level to fall only to rise again albeit to a lesser extent with addition of fresh isotonic sodium chloride. This initial flux is at a time when the cells are most vulnerable to osmotic stress and additional indirect lysis of unstable (senescent) cells is seen. Thereafter the cells settle down and remain stable for periods of time depending on the supernatant supporting medium. In the present work the supporting medium has been mostly isotonic saline since it is our policy to transfuse blood as soon after recovery as possible. Fig.4/4 shows the rise in supernatant haemoglobin over a period of days with recovered cells in saline. Clearly this illustrates the point just made and although cells remain sterile for at least 12 days if aseptically handled the added supernatant haemoglobin may prove an unacceptable burden beyond a few days (see p 175 and chapter on osmotic fragility). (Doebbler and Rinfret 1959).



Loss of haemoglobin on storage of processed units ($n = 8$) at $4^{o}C$

Fig. 4 - 4
& 4 - 22

Since mid way through this work a change was made in the method of thawing cells (p160) a series of observations were made of immediate post thaw supernatant haemoglobin with and without agitation of the blood cans in the adapted washing machine (v012). The means of the two series are shown in the Table, 4/5.

These results show that there was no significant difference between the static (non-moving) thaw and the agitated (moving cycle) thaw either in the amount of freeze thaw damage or in the quality of the final product.

Hb gm/dl	Series 1 static thaw	Series 2 agitated thaw	Significance
Post Thaw mean	0.398	0.285	t =0.4156 0.6<P<0.7
S.D.	± 0.259	0.080	not significant
Final Product Mean	0.198	0.118	t = 0.3788 0.7<P<0.8
± S.D.	0.199	0.070	not significant
	N = 74 units	N = 24 units	

Table 4/5

Comparison of Static and Agitated thawing.

EFFICIENCY OF WASH RECOVERY

In an attempt to assess the extent of recovery of frozen cells from the liquid nitrogen store a series of observations were made on the red cell mass recovered before and after freezing. Efficiency of washing procedures was assessed by measuring the levels of free haemoglobin in the supernatant at each stage of the washing process as well as haemoglobin estimations and packed cell volumes before and after freezing and thawing. Haemoglobin appearing in the supernate in each step of the process is an index of the extent of cellular damage. Percentage haemolysis is given as:

$$\frac{\text{supernatant Hb}}{\text{Total Hb}} \times (100 - \text{PCV})$$

The Table shows the haematological values for the units of blood examined in the present work: —

Table 4/46

	Haemoglobin gm/dl	PCV v/dl	MCHC g/dl	
Preglycerol	11.72	38.6	30	= 120
Glycerol	12.92	42.6	30	= 120
Post Thaw	17.6	57.6	30.9	= 116

Supernate haemoglobin studies were done on most units and the following are given as illustrations of the method of calculating recovery and the measurement of its efficiency.

		Vol	Hb gm/dl	Total Hb (Gm)	
Supernate	1	220 ml	2.16	4.75	7.205
Wash	1	330	0.45	1.48	
Wash	2	250	0.18	0.45	
Wash	3	250	0.21	0.525	
Original		220	23.9	52.58	
Packed cells Hb		10.6	PCV 31%, MCHC 34		
Supernatant Hb		0.4 gm/dl			
Post Thaw		170	24.2	41	

$Hb_{25.4}$, PCV 0.75 MCHC 34

$$\% \text{ haemolysis} = \frac{7.205\ (100 - 75)}{41} = 2.6\%$$

The efficiency of processing is shown graphically in the Fig.4/3 where it can be seen that following thawing there is a supernatant haemoglobin representing the accumulated free haemoglobin as a result of freezing and thawing. On addition of Sorbitol to act as osmotic buffer and begin the process of removal of glycerol from the cells there is a rise in supernatant due to the presence of osmotically unstable senescent cells. Thereafter the two saline washes remove the residue of supernatant haemoglobin and result in a product with acceptable levels of supernatant haemoglobin.

Based on the original cells in the donations, the calculation becomes:

$$\% \text{ haemolysis} = \frac{7.2\ (100 - 31)}{53} = \frac{7.2 \times 6.9}{5.3} = 9.3\%$$

$\therefore$ % Recovery of thawed material = 100 - 2.6 = 97.4%

and % Recovery of the original starting material

= 100 - 9.3 = 90.7%

A similar unit gave the following results:

		Vol	Hb gm/dl	Total Hb	
Supernate	1	205	0.6	1.23)	
Wash	1	260	0.195	0.51)	
Wash	2	240	0.16	0.38)	2.53
Wash	3	180	0.23	0.41)	

Original Hb 11.7, PCV 37% MCHC 32

Post Thaw 145

Hb 27.0 PCV 77 MCHC 35

$\therefore$ % haemolysis = $\frac{2.53}{35} \times (100 - 77) = 1.37\%$

$\therefore$ % recovery = 98.63% or

$(100 - 37) \times \frac{2.53}{35} = 4.5\%$ = % haemolysis of original

i.e. % recovery = 95.5% of original packed cells.

Similar results are easily obtainable for any unit of blood and one can construct efficiency of recovery charts for any unit based on the supernatant plasma levels. Since the final wash level is important in deciding if the recovered unit is fit for use, every unit issued for patients was checked until over 100 consecutive recoveries had been done with satisfaction. Thereafter most able technicians performing the recovery could be trusted to carry out the procedure in a standard manner (see technical appendix) and by visually inspecting the discard supernatant washings abnormally haemolysed units are seen. On only one occasion has this occurred and the cells were not used (p.163). Since these early studies, a change has been made to citrate phosphate dextrose as anticoagulant but this has had , if anything, a beneficial effect in that the cells appear less liable to lysis (Table 4/7). (Fig. 4/9 p161).

Donation No.	Hb mgm/100 ml Supernatant	Wash 1	Wash 2	Wash 3
CPD				
1	540	100	220	280
2	400	50	280	200
3	340	30	300	240
4	400	40	400	140
5	325	040	300	090
6	290	050	055	056
7	265	080	212	160
8	260	065	168	101
9	228	063	140	083
10	277	043	150	125
	332.5	[illegible]	202.5	147.5
ACD results from p. 151	313	67	201	160

Table 4/7

Effect of CPD anticoagulant on final recovery

Since lately the blood collected at the Regional Centre has been in CPD and not ACD a small series was done to see if the efficiency of recovery differed substantially when compared to ACD stored cells of the same age. CPD units are frozen down within 2 days as with ACD blood units. Table shows the accumulated data for some CPD blood and the figure shows this in graphic form. CPD would appear not to make any material difference to the overall recovery of frozen cells.

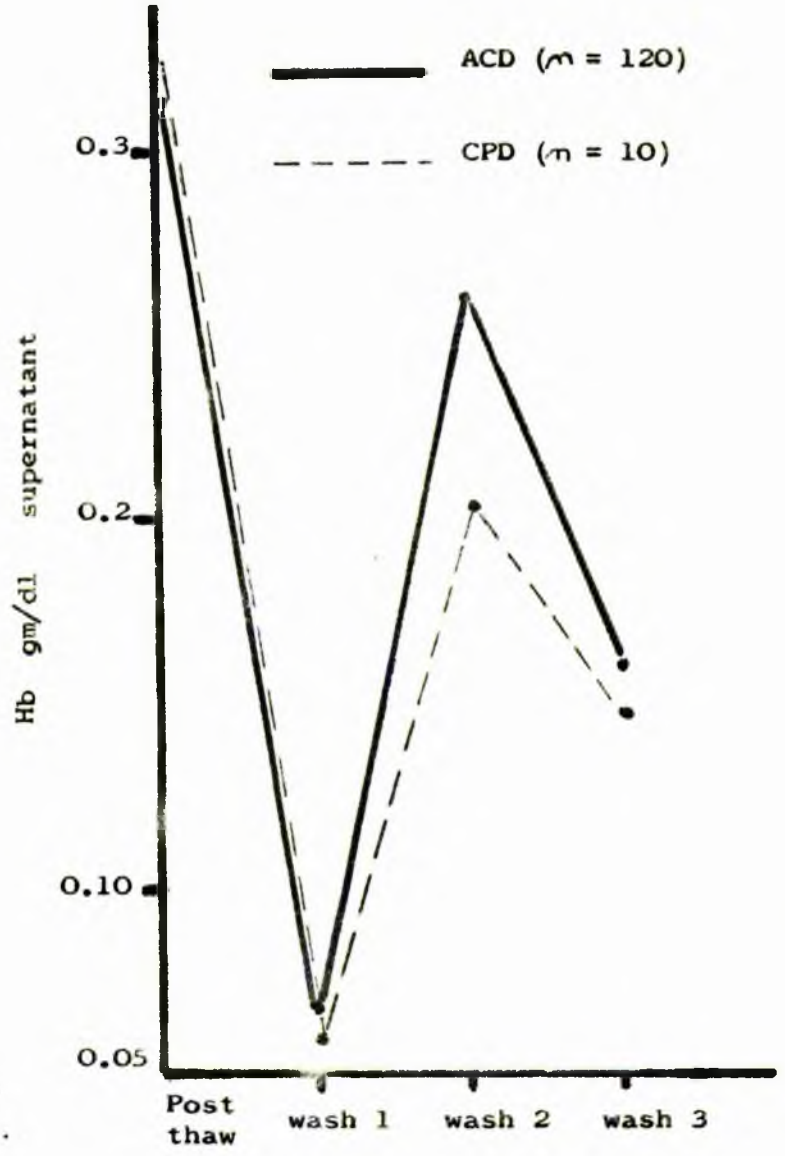
Table 4/7.		Hb gm/dl	(range)
Supernatant Post Thaw		0.350	(0.26-0.54)
Sorbitol	Wash 1	0.054	(0.03-0.63)
NaCl	Wash 2	0.236	(0.14-0.30)
NaCl	Wash 3	0.167	(0.083-0.28)

ν= 7 units Effiency of Recovery CPD Blood.

Returning to Table 4/2 it is possible using the collective data for all of the washes in the 95 consecutive units recovered to calculate the measured total loss in the supernatant for the whole unit of blood and the entire 95 units. These losses are shown in the Table 4/2

total losses as Hb gm/dl = 1.8914 $\pm$ 0.3674/units

In all cases the supernatant haemoglobins are measured after centrifugation of the collected supernate since it is realised that some viable cells do escape with the supernatant if the operator is not vigilant in preventing this loss at the time of recovery. In the processing the line of demarcation between the supernate and the cells packed at the bottom of the wash bag is not always easy to see especially with the amount of



Efficiency of recovery. ACD donations compared to CPD donations

Fig. 4/9

haemolysis in the immediate post thaw centrifuged bag. This can be avoided by visualizing the line through a bright light against a white background such as in the Pathfinder Laminar flow cabinet used for the aseptic transfers during recovery (see Pl. 4/1). To illustrate the extent of such losses 4 units were recovered and each supernatant haemoglobin measured before and after centrifugation. The initial figure represents the true supernatant + suspended cells, the second figure represents the true supernatant haemoglobin after vigorous centrifugation to make it cell free and the third figure represents, by difference, the amount of cellular (viable) haemoglobin.

Table 4/10.	Hb gm/dl	Total Hb grams
Supernatant + cells	0.925	2.127
Supernatant - cells	0.328	0.7575
Cells - supernatant	0.597	1.3695

$\bar{n}v = 4$ Haematological values during Processing.

It can be seen that unless the cells are centrifuged down in the supernatants a falsely high indication of apparent cell lysis may be found with the erroneous impression that the "efficiency of recovery" has been poor. In addition, unless care is taken, at least 1 gm of viable cells may be needlessly removed with an avoidable loss of product efficiency with nearly 50% of the "losses" being valuable red cells thrown away in the washings. It was decided to calculate the similar losses on the 95 consecutive recoveries referred to above based on measurements of the total supernatant haemoglobin for each recovery (supernatant post thaw + wash 1 + wash 2 + wash 3). Altogether the average loss

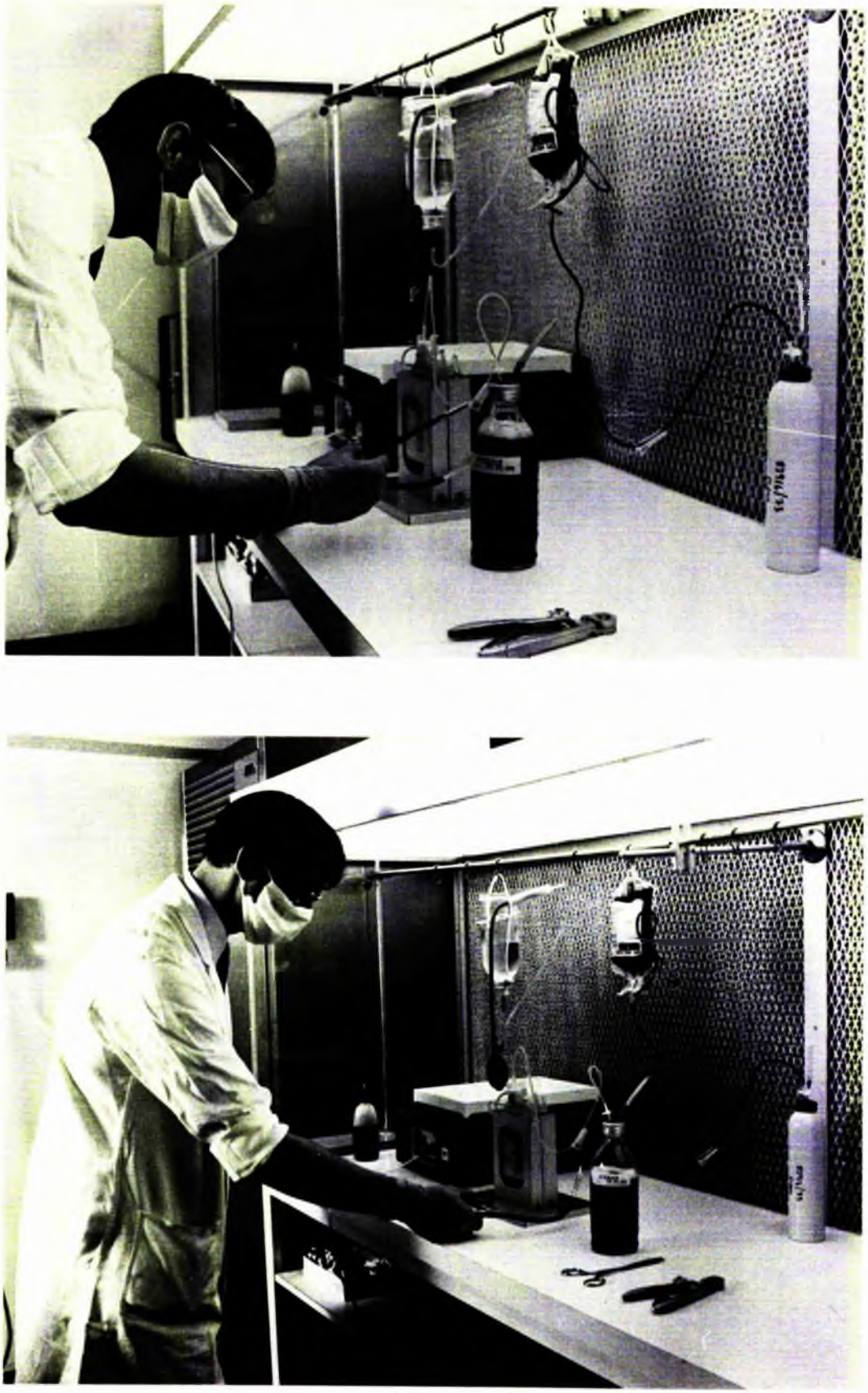


Plate 4/1. Work Cabinet.

per unit of cells (total volume of supernatant x Hb in mgm/ml) was 1.916 ± 0.762. In one extreme case 6.224 grams of haemoglobin were lost from the unit but this particular recovery was exceptional and the technician noticed that it was a poor recovery and it was not issued. Apart from this, of the 94 remaining units, losses ranged from 0.913 grams to 3.336 grams.

The graph of average efficiency of recovery shows that nearly 50% of the losses in haemoglobin is due to freezing and thawing injury.

ADDENDUM

Since this chapter was written another donor similar to that described on page 163 has been seen. This is of such fundamental importance that the circumstances are set down. The donor previously donated and his cells were frozen shortly afterwards. Six months later on recovery, the cells were not used because they showed abnormally high supernatant haemoglobin. This was not reported at the time but a note put on the donor's records. One year later he returned and again his donation was signalled ($p.v_{01,2}$) as being of interest to the frozen bank. Samples of blood in CPD were haemolysed and electrophoresed to reveal Haemoglobin Sickle cell trait (HsAS) as well as his cells demonstrating positive sickling in the sickledex test. Closer enquiry indicated the donor to be a 20 year old Zambian student. Attempts to recover his cells from the frozen bank again led to unacceptably high supernatant haemoglobin values. This case illustrates the cautionary advice given recently by Valeri (1974) who noted that 'preservation injury' might show racial differences. He

noted that 'Blood donors are not screened for enzyme deficiencies or for haemoglobinopathies which may adversely affect both the 24 hour post transfusion survival and life span of the donor red cells'. This case aptly demonstrates this and to my knowledge is the first to be seen in U.K. No details have ever been given of U.S.A. or other donor panels but since HbS is common in other parts of the world, our chance finding is of considerable interest.

Comment has already been made on the difficulties encountered in trying to obtain adequate feed back of information after frozen cells had been given (p.118). The Table shows the information obtained concerning some stable (non-bleeding) renal dialysis patients (adults). (Table 4/11)

Patient	Pretrans Hb	Post trans Hb gm/dl	Rise in Hb gm/dl	No. of Units transfused
1. E.J.	6.7	11.7	5.0	4
2. J.B.	3.0	6.6	3.6	3
3. M.McC.	3.4	4.7	1.3	1
4. J.S.	6.9	8.3	1.4	2
5. J.T.	6.6	9.3	2.7	2
6. J.W.	5.8	7.3	1.5	1
7. J.W.	5.8	7.5	1.7	1
8. P.B.	4.7	5.3	0.6	1
9. S.S.	4.0	10.10	6.0	3
10. S.S.	3.6	8.3	4.7	3
11. F.W.	3.0	6.2	3.2	3
12. R.D.	7.0	9.9	2.9	2

Table 4/11. Haemoglobin response in transfused patients.

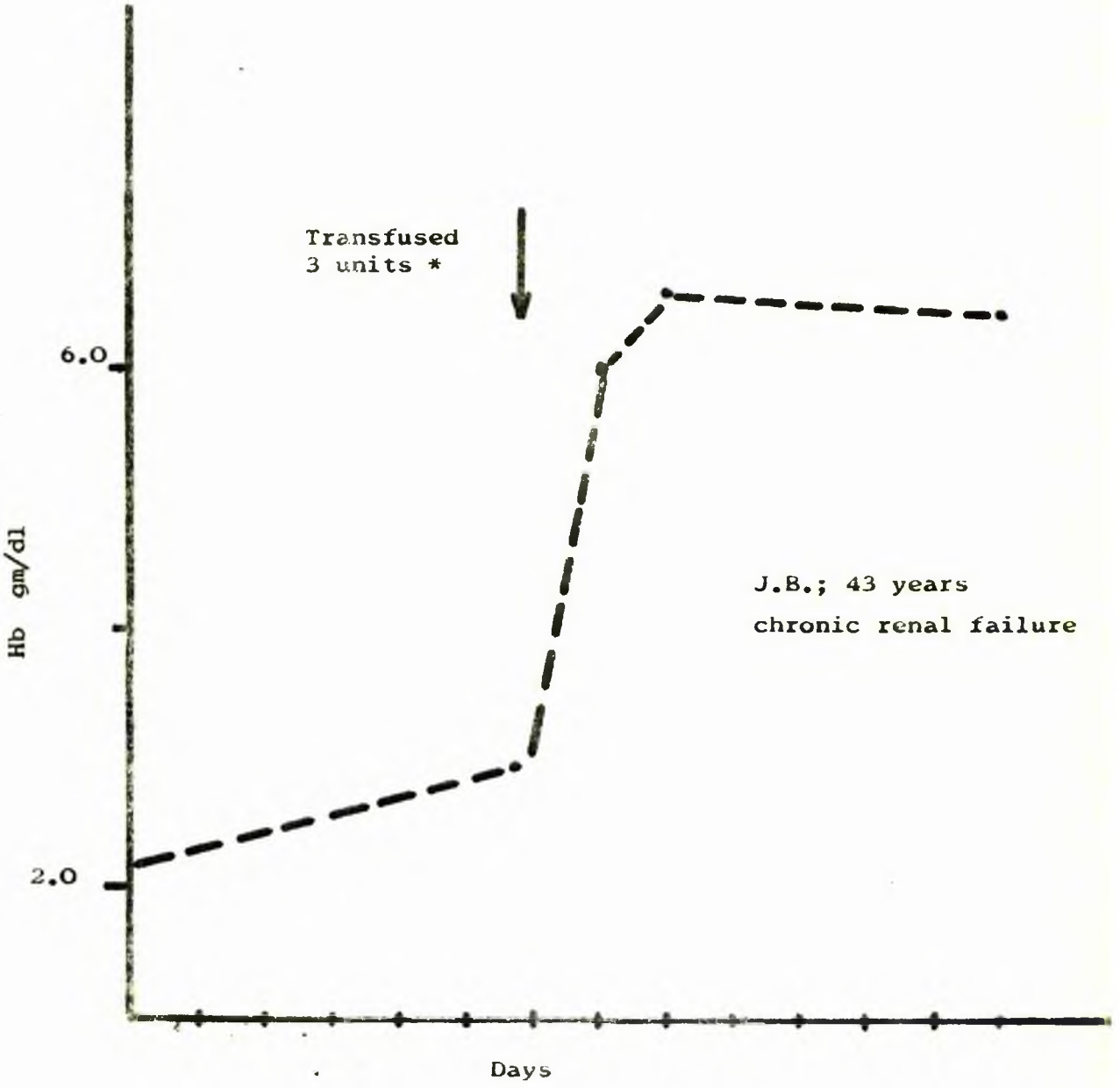
Average rise in post transfusion haemoglobin 24 hrs. after infusion 1.330 gm/dl. The total amount of haemoglobin in the transfused units was of course, known before they left the Transfusion Centre. This is shown in the Table 4/12, and diagramatically in the accompanying figures (4/13 - 18).

Patient	No. of Units	Hb in each unit gm/dl	Total Hb as red cells gm/dl	gm/units
1. E.J.	4	18.6, 12.0, 12.7, 12.9	56.2	181.59
2. J.B.	3	16.0, 18.0, 15.0,	48.9	135.3
3. M.McC.	1	18.6	18.6	50.2
4. J.S.	2	12.8, 15.4	28.2	93.0
5. J.T.	2	19.2, 19.0	38.2	

Table 4/12. Total haemoglobin transfused as red cells.

Patient	No. of Units	Hb in each unit gm/dl	Total Hb as red cells gm/dl	gm/units
6. J.W.	1	21.6	21.6	61.48
7. J.W.	1	19.6	19.6	58.8
8. P.B.	1	15.8	15.8	50.5
9. S.S.	3	17.6, 18.5, 20.0	56.1	-
10. S.S.	3	18.2, 16.9, 19.2	54.3	168.72
11. F.W.	3	19.4, 13.9, 16.0	49.3	160.20
12. R.D.	2	20.0, 16.8	36.8	108.4
	2	10.1, 13.1	23.2	64.66

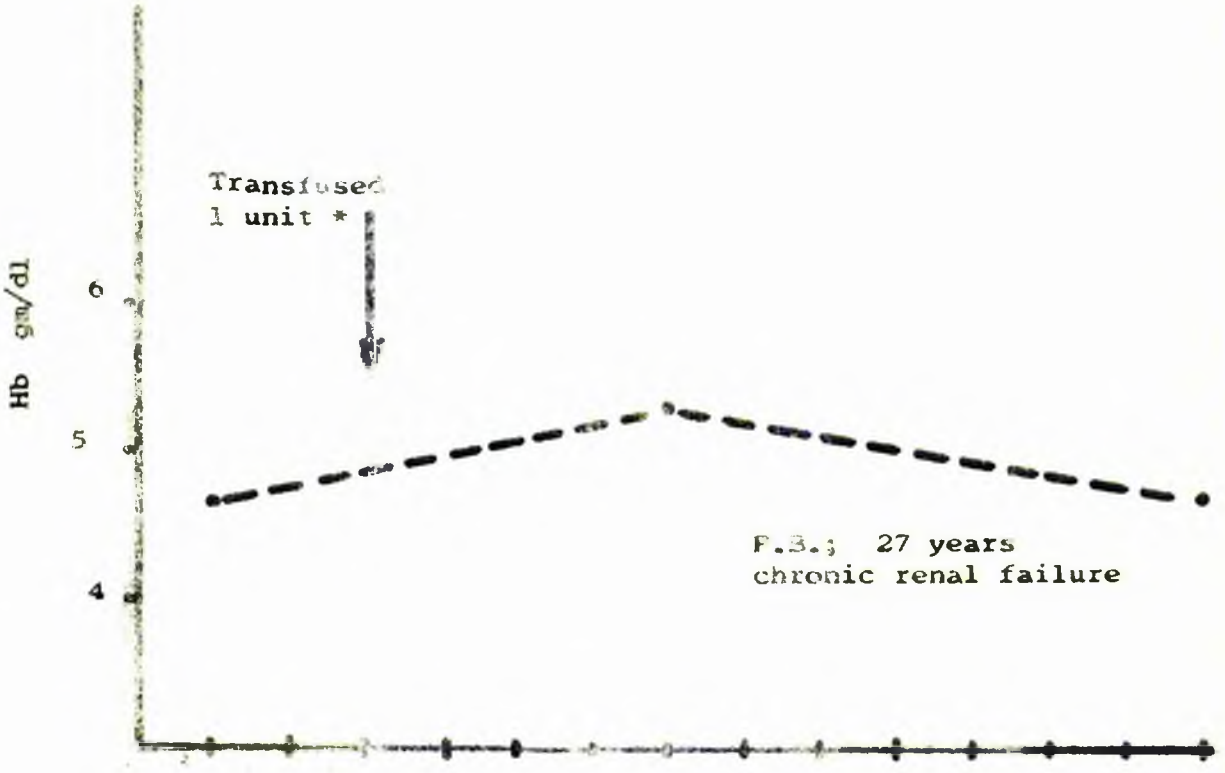
It is realised that these Tables are only for a small number of patients but they illustrate the generally acceptable favourable outcome and the difficulties in obtaining information about patients where the processing laboratory is geographically distant from the hospital in which the act of transfusion is often a minor procedure in the overall management of the patient's illness.



Response to frozen blood

*	Units	Hb gm/dl	Hb gm/unit
	82293	15.0	43.5
	86014	16.0	43.2
	86001	18.0	48.6

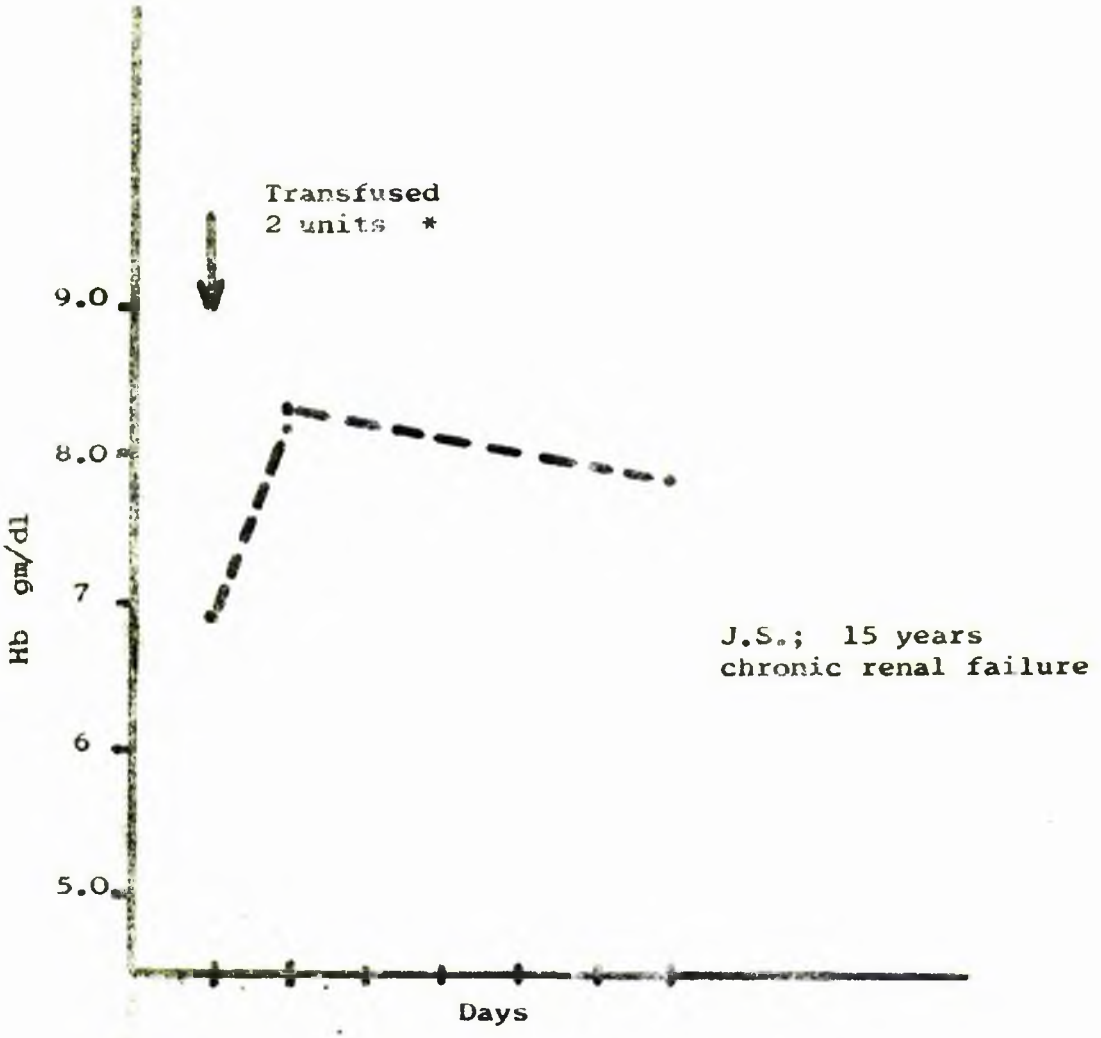
Fig. 4 - 13



Response to frozen blood

* Unit	Hb gm/dl	Hb gm/unit
25572	15.8	50.5

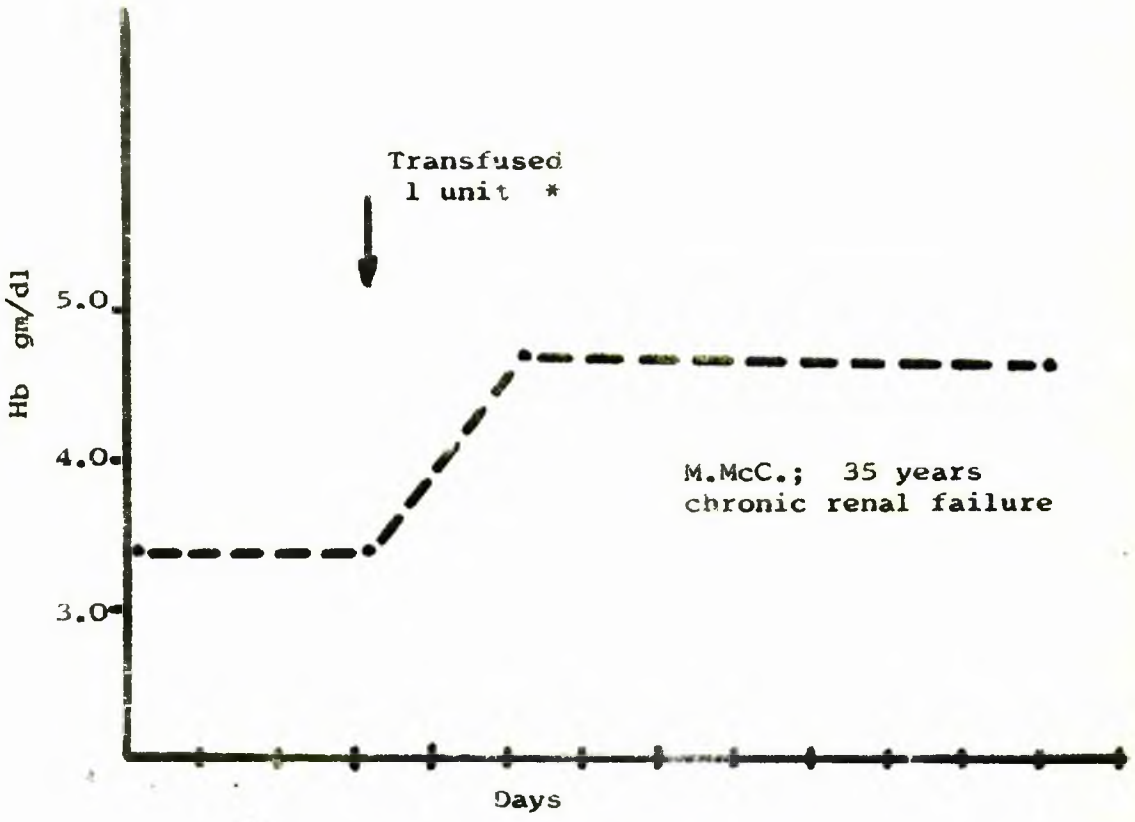
Fig. 4/14



Response to frozen blood

*	Units	Hb gm/dl	Hb gm/unit
	107380	12.8	38.4
	081865	15.4	44.6

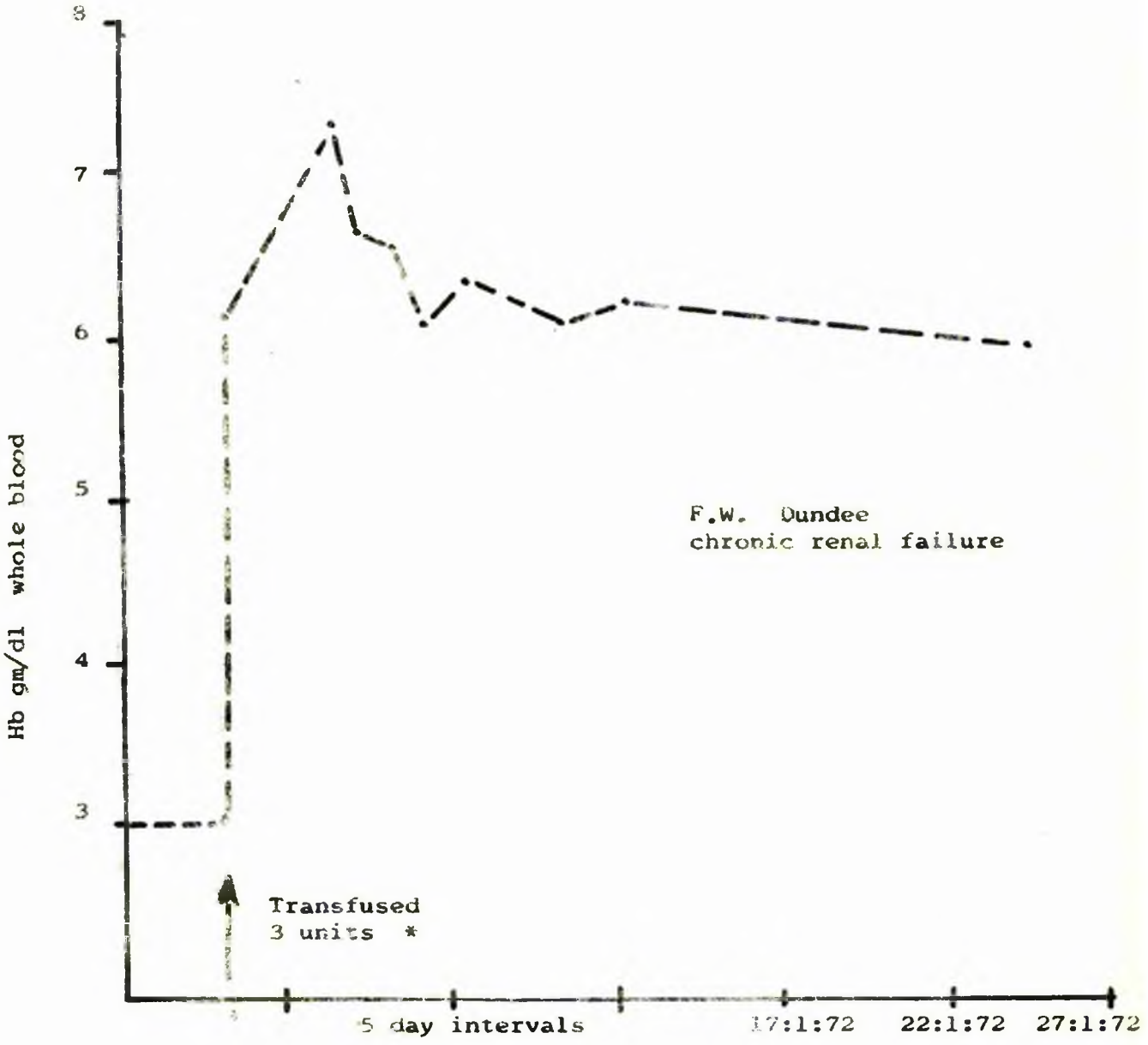
Fig. 4/15



Response to frozen blood

* Unit	Hb gm/dl	Hb gm/unit
107151	18.6	50.2

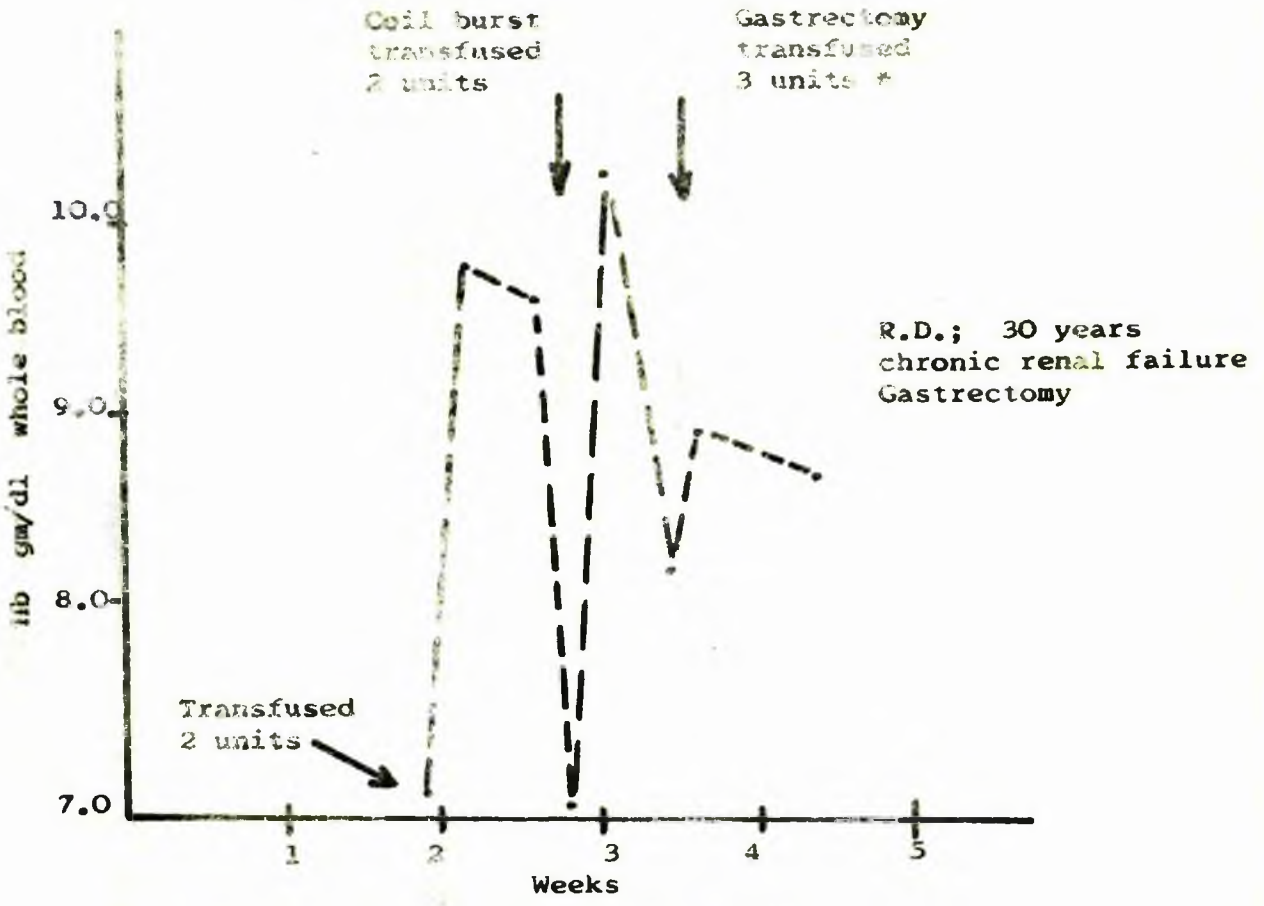
Fig. 4/16



Response to frozen blood

*	Units	Hb gm/dl	Hb gm/unit
	51516	19.4	60.14
	51509	13.9	47.26
	85994	16.0	52.80

Fig. 4/17



Response to frozen blood

* Units	Hb gm/dl	Hb gm/unit
86023	16.4	47.56
86018	20.4	59.16
86002	16.3	48.?

Fig. 4/38

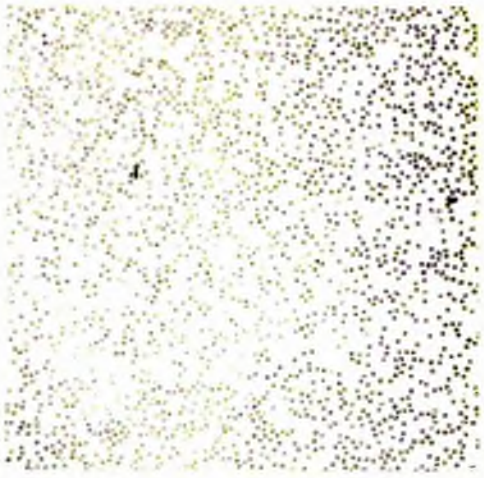
The literature is very scanty on the question of just how much red cell mass is recovered as red cells and what is their various indices such as Haemoglobin, Packed Cell Volume, Mean Corpuscular Volume and Mean Corpuscular Haemoglobin Concentrations, White Cell Count, Platelet Count and presence of alloantibodies and protein in the supernatant bathing the recovered cells. The reason for this scanty evidence is probably not that the tests were not done but that results were not recorded in an easily assimilable form. Where authors have described other functions in terms of red cells or gram of haemoglobin it is clear that some measurement must have been made but not recorded except for the calculation. Haynes et al (1960) recorded a haematocrit of up to 65%.

Measurements were made of these factors of haemological interest in a consecutive series of frozen red cells before, during and after processing using standard measuring techniques (cyanmethaemoglobin method for haemoglobin assay and coulter counter system for PCV, MCHC, WBC).

	ACD	gly	final	No. of units examined ACD	gly	Final
Hb gm/dl	11.72 ±2.35	12.92 ±1.49	17.62 ±2.95	121	120	116
PCV (v/dl)	38.64 ±6.77	42.58 ±6.6	57.64 ±7.84	91	90	112
MCHC g/dl	29.56 ±3.28	29.94 ±2.51	30.93 ±2.48	85	84	110
WBC/cubmm	4,447 ±1562	32 38 1 99	333 225	110	110	99

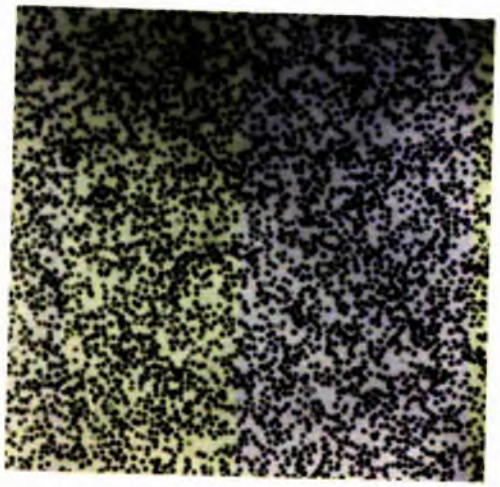
Haematological values during process.

Numerous blood films were examined of donor blood at various stages of the processing and up to 14 days in the resuspended state kept on the shelf at 4°C in the blood bank refrigerator. Red cells maintained their size and shape in all of the films examined. No special changes were noted and the slight increase in corpuscular volume noted in the immediate post wash phase was rarely (Plate 4/2) perceptable (p. 182 , osmotic fragility), thus confirming the observations of Daszynski and Maj (1968). With the assistance of Dr. Ian More, Department of Pathology, Glasgow Western Infirmary red cells were fixed in 20% glutaraldehyde and stained by a lead acetate technique after embedding and cutting sections in araldite resin. At magnifications of 30,000 frozen thawed resuspended cells showed no change in size or electron density (Plates 4/3 and 4/4). These cells were compared with the same donor's cells fixed prior to freezing. In both pre and post frozen recovered cells red cells were well preserved although in the post thawed group there were less fragmented forms and less variability. An occasional mulberry cell was noted as described by Longster et al (1972) (Plate 4/5). In all cases red cell membranes remained intact and showed no sign of any injury.



Pre-glycerol

Pre-freeze

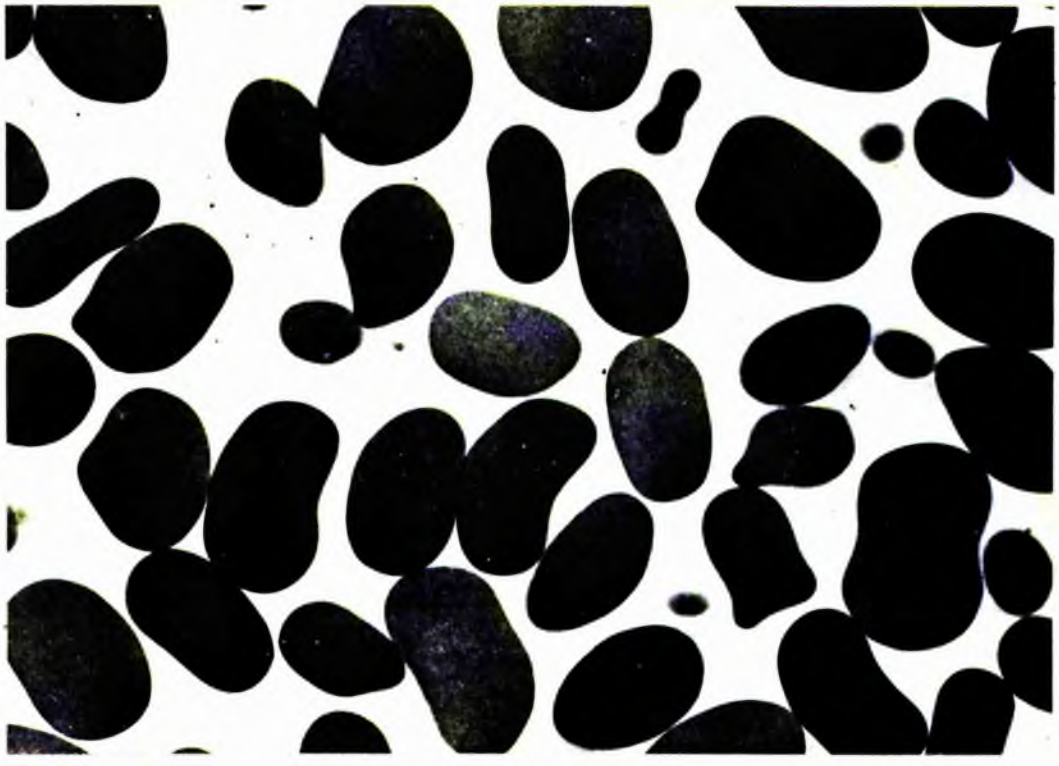


Post Thaw

Pre-transfusion

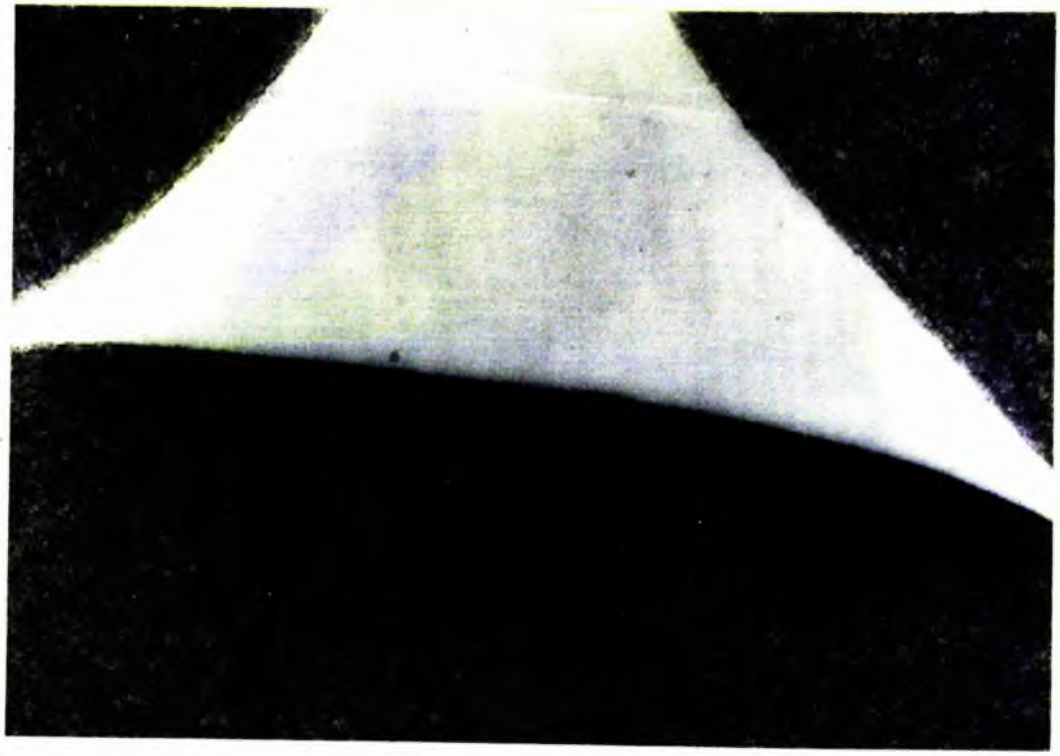
Plate 4/2. Red Cells at Different Stages of Process
x 60

ACD Donor
Blood



Same Blood after Freeze and Thaw Recovery

Plate 4/3. Electron Microscopy before and after Freeze Thaw Processing. x 8,000



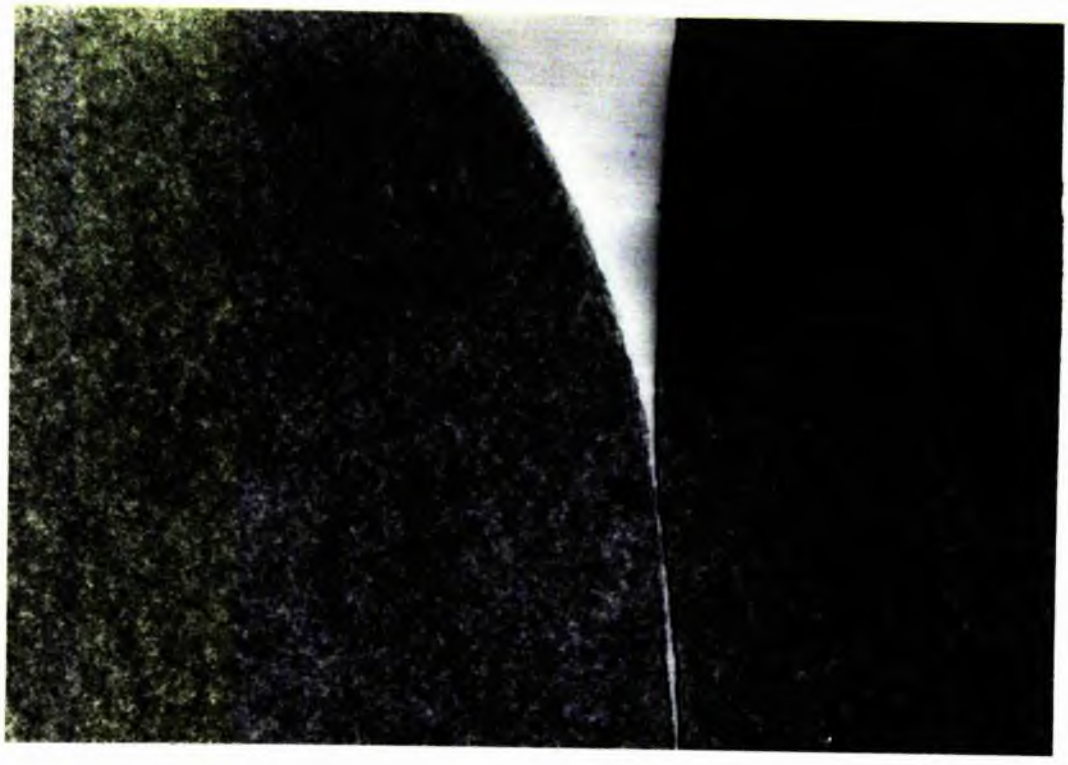
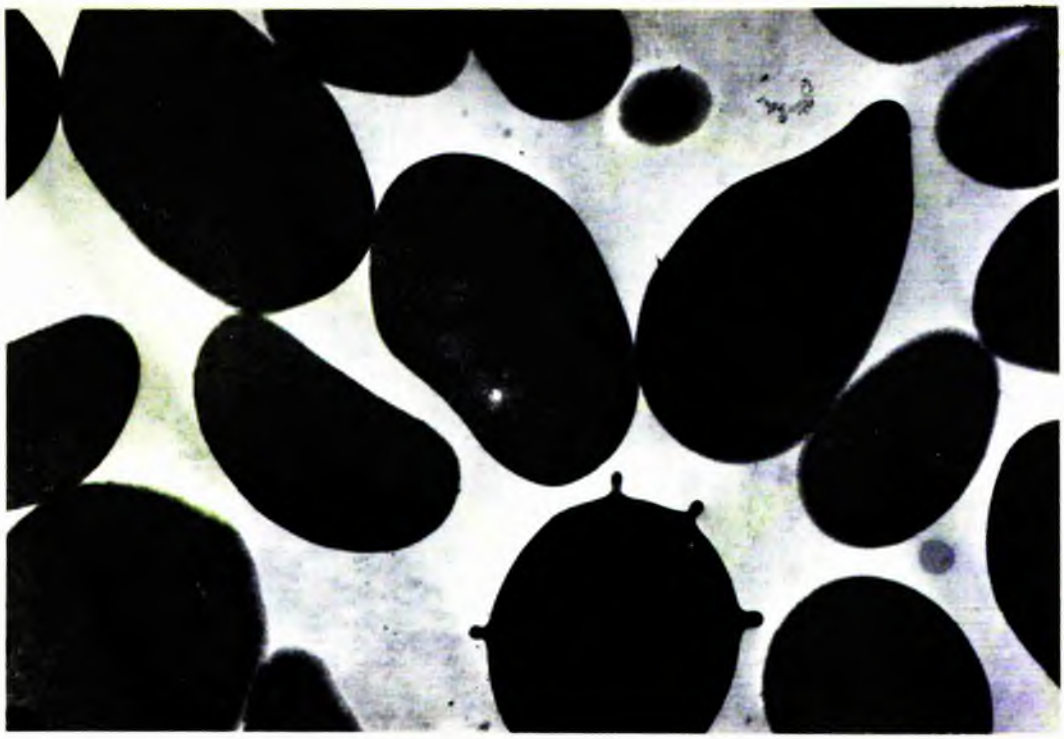


Plate 4/4. Electron Microscopic Detail of Red Cell Membranes before and after processing x 50,000



x 4000

x 50,000

Plate 4/5. Occasional "mulberry" Cell Electron microscopy

Gabrik et al (1971) showed most red cells to recover their disc form and morphology after thawing from PVP. About 5% showed some crenation and spherocytosis. Submicroscopically Vinograd-Finkel (1971b) showed by electron microscopy that cells recovered from PVP, nitrogen storage showed no abnormality in 84-86%. Beginning destruction was seen in 10-12% and 2% showed lysis.

Miura et al (1971) showed that cells recovered from glycerol (Huggins method) were occasionally crenated and that fragmentation was frequently seen in electron microscopy where a knobby or tear-drop cell could lose small portions by fragmentation yet not undergo lysis. They considered that the cells could repair their membranes and that the change was not necessarily fatal to the cell with the cell assuming a more spherical shape.

Runck, Valeri and Sampson (1968) showed that in using the Huggins high glycerol method of storing frozen cells the MCV was reduced to 55 cubic microns during the washing with hypertonic non-electrolyte solutions and to 50 cubic microns when washed with hypertonic ionic (electrolyte) solutions. During this time there is significant loss of intracellular potassium and adenosine triphosphate (Lionetti et al 1966) which is also reflected as a decreased osmotic fragility, increased cellular density and increased MCHC (Valeri, McCallum and Damon 1966; Valeri, Runck and McCallum 1967). In vivo survival of less than 70%, 24 hours post

transfusion could be statistically predicted when in vitro measurements showed MCV less than 80 cubic microns, MCHC greater than 37%, decreased osmotic fragility (25% point of hypotonic fragility at a salt concentration of less than 0.26 gm/100 ml, the 50% point at a salt concentration less than 0.23 gm/100 ml and the 75% point at a salt concentration of less than 0.20 gm/100 ml) and increased erythrocyte density as measured by the sedimentation of 10% of the erythrocyte population through the phthalate ester solution of specific gravity 1.222 or heavier.

Naradin et al (1969) emphasised the change of shape of aging red cells from disc to sphere forms, fragmentation and loss of membrane lipid, decrease in critical haemolytic volume (osmotic fragility), increased cellular rigidity and decreased cellular filterability. Restoration of ATP improved and restored in vivo survival which correlated with restoration of cell shape, filterability and viscosity. Similar results were reported by Nakao et al (1959).

Akerblom and Hogman (1974) recorded that in their low glycerol rapid freeze technique, the MCHC changed markedly during processing. In the frozen thawed blood, the MCHC was close to that of blood collected in a slightly hypotonic ACD solution. Addition of the hypertonic 3.7% sodium chloride caused shrinkage of the cells resulting in about one half of the red cell water volume being expressed due to extracellular hypertonicity (p 46). A normal MCHC was restored during the washings when the hypertonic sodium chloride solution was replaced with isotonic saline. In the

processed blood, MCHC ranged between 29.0 and 33.0 gm/100 ml (mean 30.7 ± 1.3). In blood processed by cytoagglomeration in plastic bottles or using Huggins method the values recorded were 35 and 32 gm/dl respectively.

From red cell survival studies Szymanski et al (1968 a,b) concluded that some 4 ± 9% cells in the frozen recovered infusion mass must be severely damaged and removed at the time of the actual infusion. Other less damaged cells (23 ± 9%) were removed more slowly over the next 24 hours. The former severely damaged cells accounted for in vitro losses and the latter were reflected in the age of the blood at 4°C before freezing. To avoid this they recommended freezing blood within one day of its collection.

Vinograd-Finkel et al (1971) using PVP studied the effects of liquid nitrogen freezing by electron microscopy. 84 to 86% of cells were normal, 10–15% of cells showed early evidence of damage and haemolysis and 1.5 to 2.0% showed clear evidence of damage and complete haemolysis. Lavrik et al (1971) reported on Russian experiences with low molecular weight PVP (10% mol. wt. 12,000) the average volume of erythrocytes prior to freezing of 82.4 ± 1.4 μm^3 and 87.5 ± 1.3 μm^3 on recovery at 24 hours. Most cells looked normal and about 5% showed spherocytic and mulberry forms. No abnormal combinations between plasma proteins and cells were found.

Strumia and Strumia (1965) studied the variation in mean red cell diameter when modified by their lactose/

Author	Mean Corpuscular Volume		M.C.H.C.	
	Pre freeze	Post thaw	Pre freeze	Post thaw
Gibson et al (1972)	92.2	90.0	-	-
Tullis et al (1971)	90.9	96.4	-	-
Almond & Valeri (1967)	100.2	97.5	-	-
Valeri et al (1966c)	98.2±3.7	96.4±5.8	30.7±1.1	31.4±1.2
	98.2±3.7	95.7±2.9	30.7±1.1	31.2±1.1
Akerblom and Hogman (1970)			-	30.7±1.3
Present Work			29.56±3.28	31.90±2.77

Table 4/19. Mean corpuscular volume and haemoglobin concentration.

dextran cryoprotective method and recorded a value of 5.9 μm immediate post thaw cells compared to 7.1 μm. in fresh ACD cells. Smearing and staining frozen cells showed a size similar to ACD cells. Lactose on its own uniformly reduced the size, about one third showing increased crenation and spherocytic forms. Dextran treatment gave cells which had a fairly normal appearance but some 10% were cup shaped and 10% were drop shaped. The best results were seen with lactose/dextran in combination. Resuspension in plasma showed 5-10% large spheroidal forms and a few red cell ghosts. Resuspension in saline showed a more uniform population with fewer macrocytic forms. They did not see any correlation between these various shapes and the red cell survival figures. Optimal survival was obtained with MCV of less than 100 u^3; lower values were not necessarily related to survival or improved recovery. MCV's of greater than 100 u^3 were associated with lower recoveries and survivals.

Valeri and McCallum (1965) found that defrosted cells resuspended in autologous plasma had an MCV of 93 $\mu^3 \pm 5.5$ and MCHC of 33.7% (range 33.1-36.1). Older erythrocytes survived less well than young fresh cells suggesting that older senescent cells were more unstable and susceptible to trauma of preservation, although other studies suggested a more random distribution of red cell destruction (glucose 6-phosphate dehydrogenase studies).

STUDIES ON RED CELL RECOVERY IN VITRO AND SURVIVAL IN VIVO OF GLYCEROL PRESERVED CELLS

The best test of any cells clinical acceptability is a measurement of how well it behaves and survives in vivo. In the early work on methods of freezing and recovering cells most authors concentrated their efforts in testing in vitro function and methods of improving them. As knowledge accumulated that red cells could be obtained in high amounts by technically feasible methods some turned their attention to in vivo survival studies. At the time of introducing frozen cells in West of Scotland some reports in the literature indicated that such methods yielded clinically acceptable therapeutically viable red cells. Since that time (1972) numerous further reports have appeared and although they concern a large number of different modifications of basic techniques they are shown in Table 4/2 for comparison purposes only and the reader is referred to individual methods to avoid over complication of the Table.

A study of the Table shows that most methods have resulted in survival figures at 24 hr. after transfusion which are acceptable. Szymanski et al (1968 a) used automated and manual differential agglutination techniques on cells recovered from a -80°C high glycerol (Huggins) type bank. The auto analyser was used to perform quantitative reproducable separations of mixed red cell populations with anti-A, anti-B, anti-CD and anti-M in a medium containing haemagglutination

Tullis et al (1958)	80	84.6 (4-98). ∿ = 33 T/2 = 30
Haynes et al (1960)	82%	90 (84-96%). ∿ = 3 T/2 = 30
Krijnen et al (1964, 1965)	97.9	99, 94.5, 94
Prins and Loos (1965)		97.5 (T/2 30) 100 (T/2 30) 97 (T/2 28)
Sloviter and Kavdin (1965)	70	70
Strumia and Strumia (1965)		
a	98.5	69.14
b	98.57	
Valeri et al (1965 a,b)	20 ± 6.3% losses	85 ± 7.5
Almond and Valeri (1967)	26 ± 9.4% losses	-
Pert et al (1967)	"no reduction compared to fresh blood"	
Daszynski and Maj (1968)	99.2 (98-24-99.6)%	
Gibson et al (1968)	-	84 (79-88)%
	-	91 (83-97)%
Szymanski et al (1968)	26 ± 4% losses	90 ± 20%
Runck and Valeri (1969)	96.6 ± 1.9 98.5 ± 0.6	
Valeri and Runck (1969)	79.0 ± 4 79.1 ± 86	73 ± 17.5 73
Valeri and Runck (1969b) Rowe	- stored for 31½ hrs in glucose saline before transfusion	86 ± 5
Akerblom and Hogman (1970, 1974)		90-95% 87-96% 24 hr 48 hr 99.0±5.2 92.2±5.5 89.4±4.4 24 hr 48 hr 99.8±3.3 91.0±3.4 88.0±4.6
Huggins (1970)	8.5 ± 5%	87-95%
Krijnen et al (1970) a	95 (91-98)%	93 (89-95)%
b	96 (94-98)%	
Pert and Schork (1970)	"as good as ACD fresh blood"	
Rowe et al (1970)	90%	96 ± 1.5 T/2 28-30
Valeri (1970) Rowe	4-10% losses	85% (batch wash) 85% (ADL bowl)
Huggins and Grove-Rasmussen (1971)		
Tullis, Tinch and Latham (1971)	90% (85-94%)	77-99%
Button et al (1972) Norma	-	81 ± 8.6% at 21 days
Gibson et al (1972)	87.4%	87 (80-93)%
	92.64%	86%
Meryman and Hornblower (1972)	98	85 (81-90)%
	98	85 (81-90)%

Table 4/20. Recovery and in vivo survival.

Table 4/20

Author	In Vitro Recovery	In Vivo Survival (24 hr)
Orlina et al (1972) a	88	89.8 (89-92)%
b	88	-
c	88	86.8 (78-94)%
Runck and Valeri (1972)	-	94.7 ± 2.8% 94.4 ± 1.0%
Akerblom and Hogman (1974)	-	97.0 ± 5.2 99.8 ± 3.3
Derrick et al (1972)	not given	
Seidl et al (1970)	94.1 ± 2.9	86.2%

supporting additives. The mean life span of transfused cells was 96 ± 17 days. They further reported (1968b) survival studies in normal and anaemic though stable recipients that as much as 16 ± 7% of frozen cells were non-viable and removed from the circulation within a few days of transfusion (similar to ACD blood stored at 4°C for more than two weeks). The fresher the blood at the time of freezing the better the survival in vivo on recovery. Thereafter the viable cells were removed at a rate of 1.1 ± 0.2% per day giving a mean life span of 92 ± 17 days (range 57-126 days) which was similar to their findings with conventional ACD stored blood up to 14 days old (86 days, range 70-109 days). These findings confirm those of Jones et al (1957), Tullis et al (1958) and Szymanski and Valeri (1971), that red cells circulating 24 hrs after transfusion have normal long term survival when transfused into stable (non-bleeding) anaemic recipients). Huggins (1970) stated that his high glycerol/cytoagglomeration method achieved an average 85 ± 5% recovery of which 87 - 95% survived in vivo in a perfectly normal fashion. These findings were independently recorded by Valeri (1968) and Perrault et al (1967). Vinograd Finkel and Kiselev (1971) reviewing Russian work believe that low glycerol rapid freeze gives best results similar to those reported in the Table and quote in vivo survivals of 88-95% (T/2 30 days) with a shelf life post thaw of 6 days which might be extended with supplementary adenine addition to 12 days. Pert (1969) reviewed studies on bowl and manually washed recovered cells from a low glycerol storage bank. If blood was frozen within 24 hrs

of collection, the average loss of cells was 0.5% as immediate post thaw injury and removal of damaged and senescent cells. A further 0.25% loss occurred during the actual washing due to cells trapped in the bowl and transfer tubing as well as in the discard. Sometimes with careless attention to detail further losses are to be expected up to 2%. Overall recovery was over 95% (see p.176) and post process supernatant haemoglobin was 10-30 mgm/100 ml.

Tullis et al (1958) estimated the following losses: collection glycerolization, freezing and deglycerolization 10%; intact cells left on walls of containers and plastic tubing 5%; intact cells sacrificed for bacterial cultures and other assays 3%; intact cells sacrificed for typing, cross-matching and serology 1%; haemolysis during the frozen state (-80°C) 0.5%. They considered that in the immediate post thaw period there was a selection of a population of cells which had not been irreversibly and randomly damaged during collection and processing. Strumia (1964) in an isolated and undetailed report is quoted (Lund 1964) as presenting evidence to the 28th Scientific meeting of the Protein Foundation in Cambridge and Massachusetts that only 70% of cells are retained in the circulation after 24 hrs - the loss occurring in the washings prior to transfusion or by allowing the circulation to be its own [illegible] of damaged cells. He is noted as indicating that the remaining surviving cells are lost at a lesser rate than would occur with fresh red cell transfusions.

de Verdier et al (1963) presented some evidence to

suggest that young red cells could survive freeze thaw injury better than aged cells although there was also about 50% of cells showing random non selective destruction.

Storage post recovery for up to 14 days at 4°C suspended in buffered saline showed a steady incremental haemolysis in the cells up to 400 mgm/100 ml with a commensuate fall in cell potassium. This loss of cell potassium is normally present to about 10% immediately post thaw and has been held to be a district advantage of frozen cells in uraemic patients since the cells rapidly regain potassium in vivo after transfusion and therefore can be said to behave like a "potassium sponge" although Rowe et al denied its existence by their acute experiments (1970). Of 55 patients, renal, hepatic and cancerous, transfused by Pert and Schork (1970) none showed any adverse effect. All reported the expected rise in haemoglobin and haematocrit. Red cell survival was the same as fresh ACD conventionally banked blood. With Lundberge in 1968 they reported that processing losses seldom exceed 3%. Transfusions in dialysis patients had been well tolerated and patients haptoglobin remained normal suggesting that there was minimal haemolysis of frozen recovered cells in vivo. Studies in heart lung by-pass situations had revealed no upset in electrolytes although they pointed out the danger of (a) trying to freeze potassium depleted cells which were too old for freezing and (b) resuspension in hydrolysed sucrose which because of the degredation to two smaller sugar molecules (glucose and fructose)

there is a dehydration effect on the cells with excessive potassium loss. Both of these situations could result in serious hypokalaemia. Krijnen et al (1970) reported recoveries of more than 90% and in vivo survivals well in excess of the 70% minimum limit.

STUDIES ON OSMOTIC FRAGILITY AND POST THAW STABILITY

Few studies have been done on the osmotic fragility of cells recovered from frozen storage. It has been generally assumed that other biochemical tests of red cell stability (ATP, 2,3 DPG, K^+, Na^+ in vitro saline stability etc. q.v) have been sufficient to prove that any danger due to freezing and thawing must be minimal. Valeri (1966) showed that there was some decreased osmotic fragility which was directly correlated with post transfusion survival in vivo. Mollison and Young (1942) found that cells stored at -20°C in citrate/sucrose had a decreased survival and decreased osmotic fragility. Prins (1965), Krijnen et al (1964), Loos (1965), Rowe et al (1968, 1970, 1971), Jenkins and Blagdon (1971) showed that frozen recovered cells are slightly less stable than unfrozen cells but that rapid recovery occurs when the cells are returned to more physiological conditions such as in transfusion into a recipient. Hanson et al (1972) found that in cardiopulmonary by-pass, recirculation of frozen recovered cells on the pump oxygenator for 3 hours revealed them to be satisfactory but slightly less stable than in whole blood. Haynes et al (1960) reported the effectiveness of frozen cells in a pump oxygenator priming (3000 ml) during open heart surgery during a recirculation lasting 3 hours at 32-37°C. The loss of red cells only amounted to a maximum of 15 ml in 3 patients - an average loss of 0.45%. The supernatant haemoglobin liberated by the trauma of the pump during the first 30 minutes was less than in a control study using ACD blood. Hanson et al (1972) showed that washing

frozen cells with plasma was better than saline alone and that the cells showed less osmotic instability as measured by osmotic fragility and free haemoglobin release into the supernatant.

Osmotic fragility studies in the present study were performed on saline resuspended recovered red cells as well as in frozen samples of ACD donations at storage intervals of 1-10 days at $4^{\circ}C$.

Table 4/21	Lysis commences	Lysis complete	M.C.F.
Frozen cells recovered 1 day	0.85%	0.40%	0.51%
Control sequestrene	0.53%	0.30%	0.42%
Frozen cells 6 day storage	0.72%	0.52%	0.56%
6 day old ACD	0.48%	0.40%	0.48%
Control sequestrene	0.53%	0.30%	0.44%
Frozen cells recovered 10 days	1.0%	0.48%	0.74%
10 day red ACD	0.68%	0.36%	0.45%
Control sequestrene	0.52%	0.30%	0.52%

From these results and the graph of osmotic fragility it can be seen that the frozen red cells are nearly normal on the first day of recovery but with a tendency to instability if one considers the mean cell fragility as being the point at which 50% of the cells are haemolysed. With time the cells become more and more unstable until at 6 and 10 days post thaw they are in a very bad condition and 50% lysis occurs in solutions near to normal tonicity (0.56% and 0.74% NaCl). This would strengthen the argument for resuspension of frozen cells in autologous plasma (Krijnen et al 1964), or a metabolically active resuspension medium such as sucrose

Fig 4/21a.

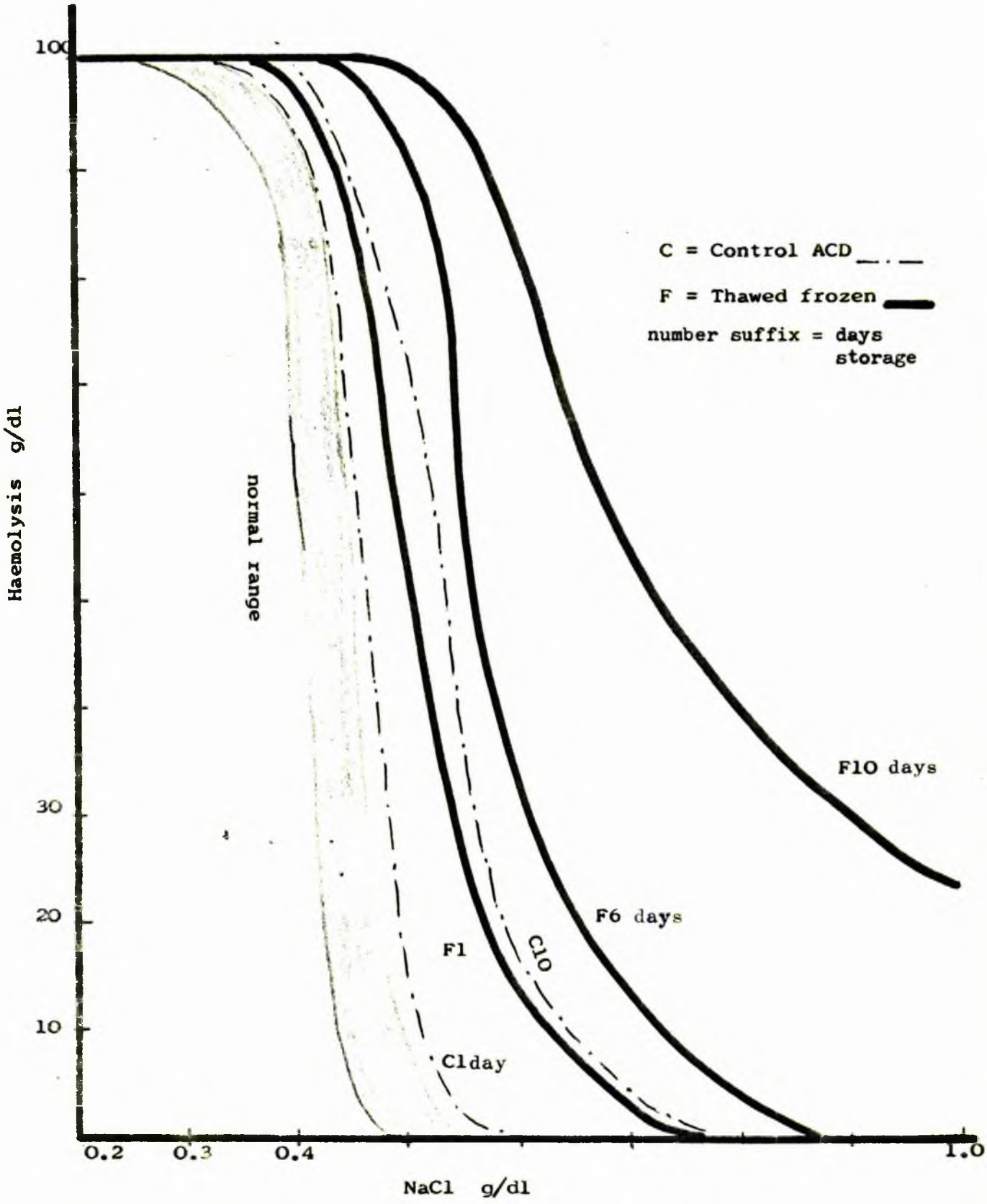
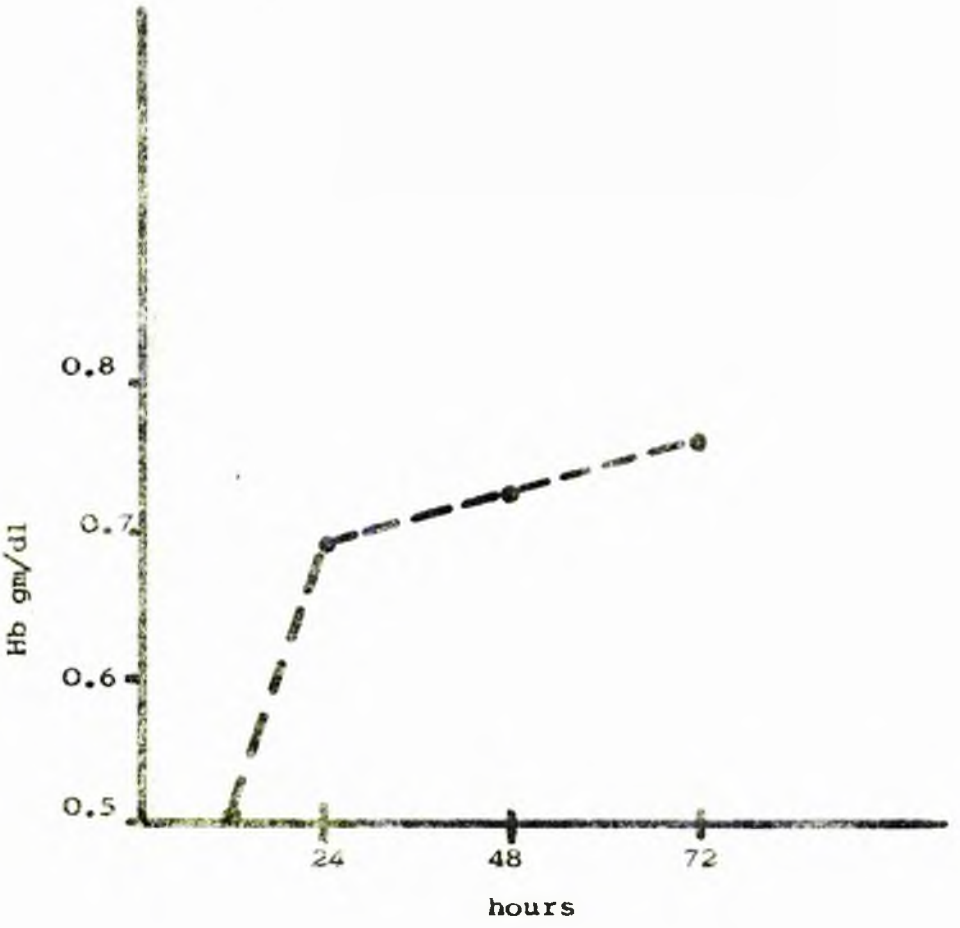


Fig 4/21a. Osmotic fragility of saline suspended frozen cells after various storage intervals at 4°C

saline where it is established that the sugar can provide enough substrate to re-energise the cells by additional glycolysis and restoration of ATP (p135), which in turn would restore osmotic stability to the cells by re-establishing the ionic equilibrium and membrane integrity. Valeri and Runck (1969a) found that after 72 hours storage in saline glucose (50 mgm/100 ml) supernatant haemoglobin and cellular potassium levels reflected the capability of some resuspension media to delay the accelerating spontaneous haemolysis for a few days (fig.4/22). Although Valeri and Bond (1966) found acceptable post transfusion survival of cells stored for up to 6 days in saline medium at 4°C (p 92) and Haynes et al (1960) recommended up to 10 days in a 5% albumin medium and 14 days in autologous plasma. When recovered red cells are inadequately preserved or there is undue delay in storage before use, the loss of damaged cells occurs immediately on transfusion into the patient's isotonic plasma. As many as 80% of damaged unstable cells are removed within 10 minutes (Strumia 1965; Valeri, McCallum and Danon 1968). Akerblom and Hogman (1970, 1974) recommend resuspension in ACD or ACD - adenine plasma, either autologous or homologous, if more than 8 hours delay was anticipated before infusion. Better stability is achieved (up to 14 days) by resuspension in a colloid medium such as albumin solutions, oxypolygelatin or autologous plasma (Tullis et al 1958, 1963, 1971; Valeri 1966; Almond and Valeri 1967 a,b; Ganshirt and Seidl 1974) although even here there is a steady decline in red cell viability and metabolic integrity as measured by ATP (Lionetti et al 1966).



Loss of haemoglobin on storage of processed units (n = 8) at $4^{\circ}C$

Fig. 4 - 4
& 4 - 22

Post Thaw Stability

At the end of recovery resuspended cells in saline were prepared as a 1 in 50 dilution and supernatant haemoglobin determined immediately and after 30 minutes incubation at room temperature. Using the Coulter Counter Model ZF6 additional observations were made of red cell indices. These results are shown in the Table. (4/23).

Table 4/23

	Time 0 minutes				Time 30 minutes			
Unit No.	RBC $0^{12}/l$	MCV fl	MCHC g/dl	Supernate mgm/dl	RBC $0^{12}/l$	MCV fl	MCHC g/dl	Supernate mgm/dl
043792	0.23	78	47	0.006	0.23	86	39	0.006
097076	0.18	86	43	0.012	0.19	88	40	0.012
087711	0.18	83	43	0.010	0.19	94	43	0.018

From these results it can be seen that the cells resuspended in isotonic saline upon recovery show very acceptable post recovery osmotic stability. Osmotic fragility curves of these same units were prepared by the method of Parpart as outlined by Dacie (1963) immediately on post thaw recovery and saline resuspension and after 24 hours shelf storage at $4^{o}C$. These results are shown in the attached graphs where it is clear again that recovered cells have a nearly normal osmotic fragility which is maintained for at least 24 hours.

Glucose Utilization

In this section it seems appropriate to comment on glucose utilization by post freeze recovered red cells. Rowe et al (1970) and Derrick et al (1972) showed that recovered red cells can quickly reinitiate glycolysis and utilize glucose (within 30 minutes) as effectively as non-frozen control cells of the same age. For this reason they recommended the addition of 400 mgm/glucose

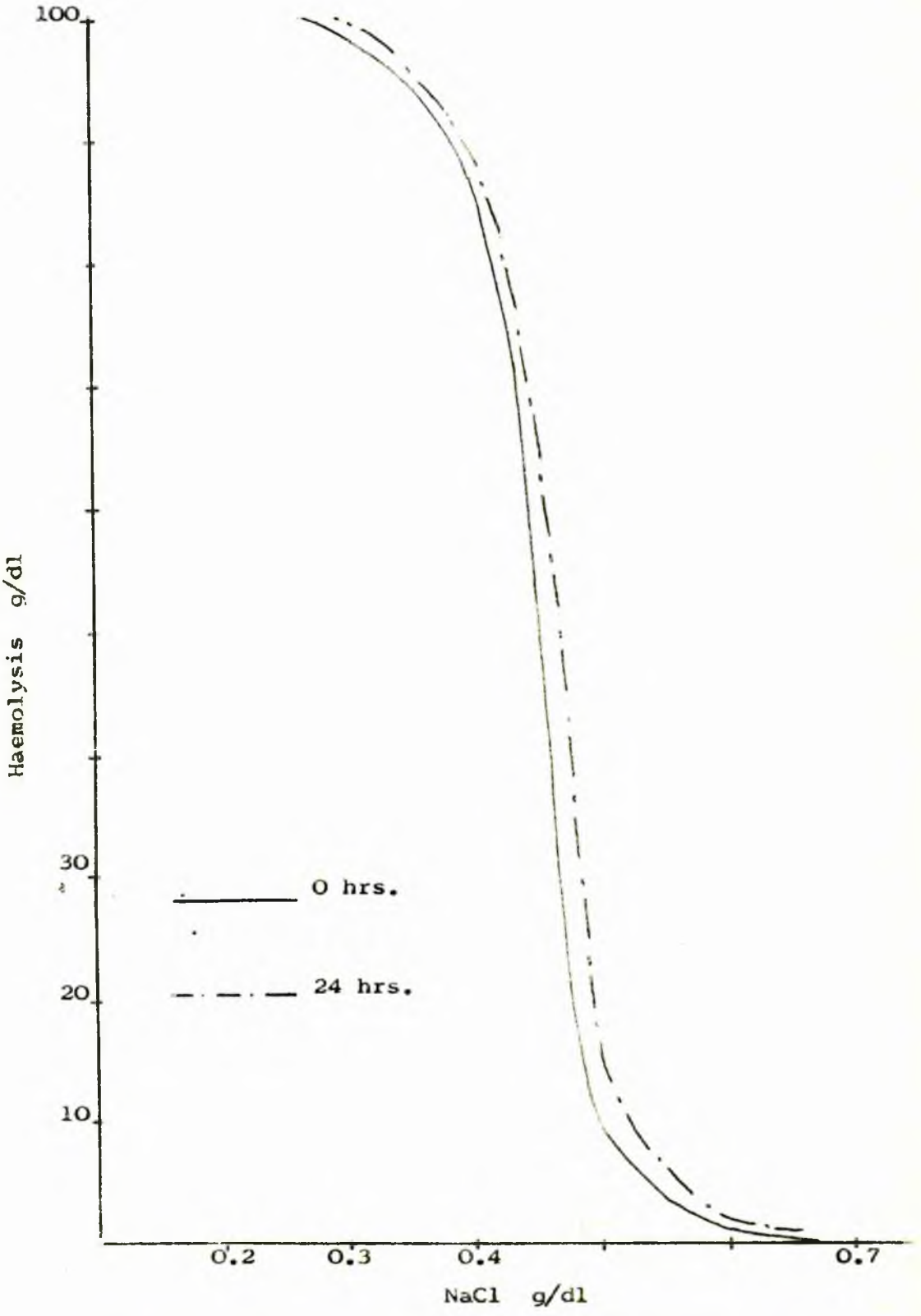


Fig. 4/23 Osmotic fragility of saline resuspended cells immediately and after 24 hours post thaw storage at 4 °C

to 100 ml suspension medium to replenish the energy reserve substrate which would, of course, be washed away in the recovery washing procedure. Tullis et al (1958) and Rowe et al (1970) also recommended 400 mgm dextrose in albumin and later recommended 100 mgm glucose/100 ml in autologous ACD plasma to prevent accumulation of supernatant haemoglobin. Krijnen et al (1964) added glucose to the resuspension medium to improve intracellular production of ATP and restore the slight losses noted on storage. The Table shows published figures on glucose utilization by frozen recovered red cells (Derrick et al 1968).

In passing we note that Chaplin et al (1954) found normal red cells consumed 0.62 mgm glucose/100 ml/day at 4°C; 0.084 mgm/100 ml/day at -10°C,; 0.008 mgm/100 ml/day at -20°C and "probably nil" at -79°C.

Author		μM/gm Hb	
		Pre-freeze	Post freeze
Huggins (1965)			4.5 ± 0.6 gm/100 ml
Shields (1968)	ACD	1.05	-
Tullis et al (1971)	ACD	391.0 mgm/ 100 ml	6.6 mgm/100 ml in saline suspension
Gibson et al (1972)	ACD	1.05	0.95
	CPD	1.13	0.86

Table 4/24. Glucose utilization of recovered cells.

Reference has already been made to the levels of supernatant haemoglobin in the resuspension medium of frozen recovered red cells (p92). Clearly it is a measure of the extent of cell injury during preparation, freezing, storage, thawing, post thaw recovery and post thaw shelf life at $4^{o}C$. Since it is relatively easy to measure haemoglobin in solution by the method of Cripps or some modification of the cyanmethaemoglobin method, many authors have confirmed their studies on post thawed cells to measurements at various stages and time intervals.

Table 4/25 shows the accumulated data from the literature by a variety of techniques.

This Table is somewhat complicated and shows wide variations in different published series due mainly to differences in freezing and thawing, high or low glycerol, slow or rapid freezing, mechanical or gaseous cooling, batch centrifugal washing, agglomeration recovery of cells, continuous centrifugal washing of cells by various machines and composition of resuspension media. Huggins calculated (1970) that recipients of frozen cells usually receive between 10 and 20 mgm of free haemoglobin per unit - an amount frequently found in ACD banked blood 21 days old. Meryman (1970) was in favour of a supernatant haemoglobin in each unit transfused of no more than 300-400 mgm. Tullis et al (1958) recorded rises of up to 30 mgm/dl in the immediate post transfusion period in 33 recipients.

In order to determine the haemoglobin supernatant figures for our own series, estimations were done on samples of supernatant ACD plasma before

Author	Pre-freeze	Post Thaw	
Tullis et al (1958)	-	150	240 at 1 week 300 at 2 weeks
Krijnen et al (1964)	10	50	100 at 7 days 150 at 14 days 200 at 21 days 4°C storage
Prins and Loos (1965)	10	50	
Valeri et al (1966) 1	202.0±49.9	470±182.3	per unit in (autologous plasma up to 11 days
Perrault et al (1967) (Huggins) ACD		159.0 (77-339)	
Daszynski and Maj (1968)	-	30	
Shields (1968) ACD	4		
Runck and Valeri (1969)	-	466±162	
Valeri and Runck (1969 a)	-	304±150 450±120	after 4 days at 4° in glucose saline
Valeri and Runck (1969 b)	-	457±158 973±445	after 3 days at 4° in glucose saline
	-	187±93	
Krijnen et al (1970)	-	45 (30-75)	
	-	42 (30-50)	
Button et al (1972) ACD	1.3 (1 day)	-	
Gibson et al (1972) ACD	3.0	136	
CPD	4.0	44	
Meryman and Hornblower (1972)	5.7±4.5	71±38	
Orlina et al (1972) a	132.5	119.4	
	137.8	124.8	
	131.1	129.5	
Akerblom and Hogman (1974) batch wash	-	75±30	glass bottles 400-1200
agglomeration	-	175	plastic bags at 4°C
		98±34	plastic bags at 24 hr.
Valeri (1974) 1	-	233±154)	430±101) 24 hr.
2	-	227±131) immed-iate	501±317) at 4°C
3	-	333±113)	499±348)
4	-	224±100)	362±51)
Almond and Valeri	56.8±74.8	285.2±121.6	
Huggins and Grove Rasmussen	-	125	
		100	agglomerator
Runck and Valeri CPD/LG ACD	- (1 day)	15.7 (calculated)	489±45 residual total

Table 4/25. Supernatant haemoglobin by various methods.

TABLE 4/25 (cont'd.)

Author	Pre-freeze	Post Thaw	
-80/HG	- (1 day)	11.6 (calculated)	463±85 residual total
	- (1 day)	457±158	total per unit
	- (3 days)	973±445	total per unit
Seidl et al (1970)	-	100	

LG = Low glycerol method

HG = High glycerol method

glycerolization, red cells during glycerolization and in the final wash and preparation of cells for issue to patients at the moment of despatch. (Table 4/26. In addition release by unstable cells was examined in final cell products at varying storage intervals at 4°C to see how long it would be before the free haemoglobin reached unacceptable levels. (This should, of course, parallel the osmotic fragility figures, figures for release of potassium from red cells etc.).

Table 4/26 Hb mgm/100 ml supernatant

No. of Units	Pre-freeze	Post freeze final Product ~ = 100
100	25.24±13.3	182.0±95.0

These results are very satisfactory when one recalls that the total supernatant volume in each pack prepared ready for use is less than 100 ml so that no patient should receive more than 200 mgm free haemoglobin in the supernatant resuspension fluid. (70 ml see p 153).

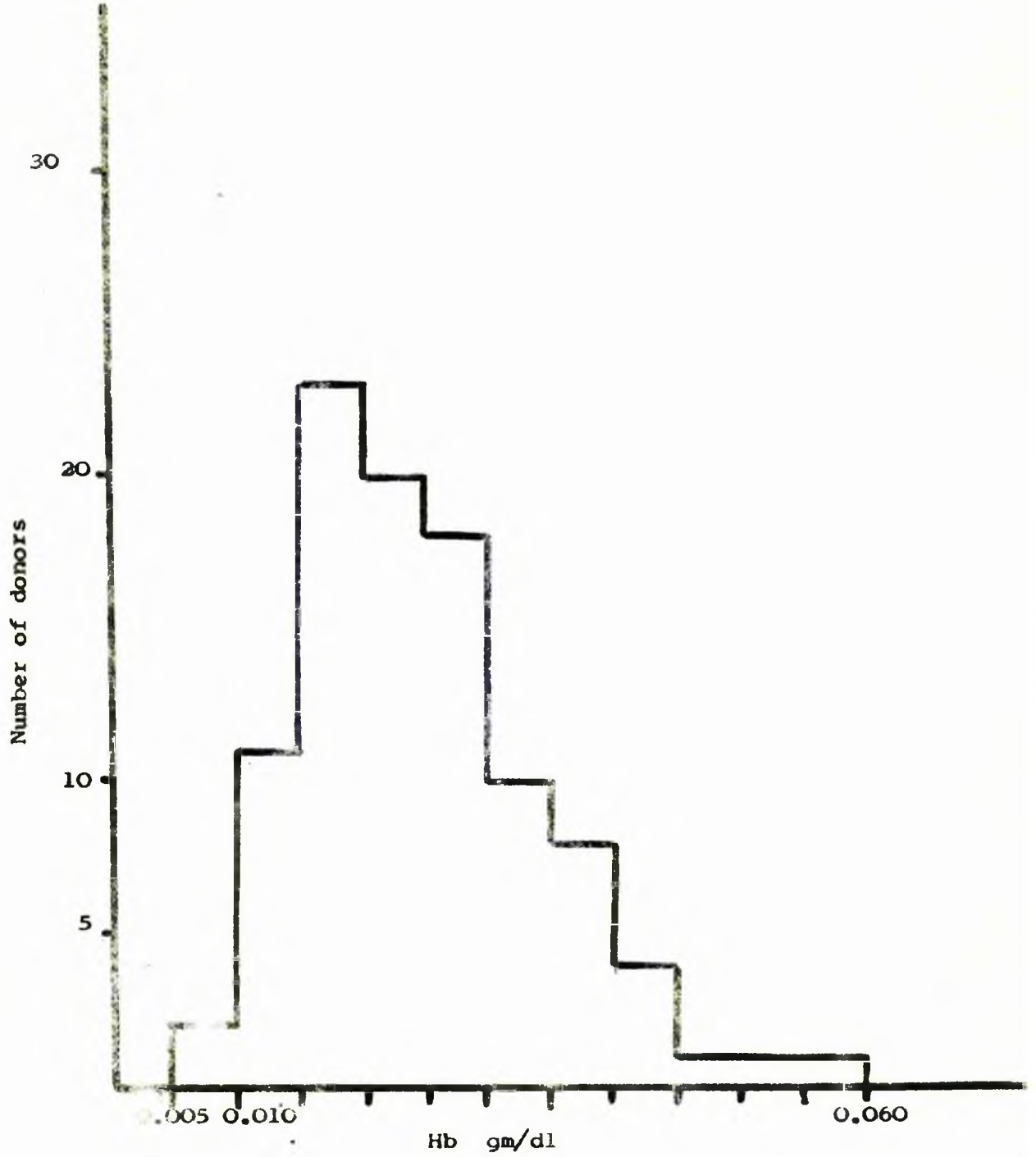
Since various workers would like to extend the shelf life of recovered frozen cells beyond 12 hours a series of 8 specimens were examined at intervals after recovery. The results are shown in the Table and Figure 4/22.

Table 4/27 Hb mgm/100 ml supernatant

Time after final preparation:

12 hours	507
24 hours	690
48 hours	725
72 hours	764

These results indicate the previously noted (p.186) accelerating haemolysis which occurs in vitro with



Plasma haemoglobin levels of

Normal blood donor samples

Fig. 4/28

recovered cells suspended in saline only (Tullis et al 1958, Seidl 1970).

It is to be noted that the "normal" pre-freeze values of plasma haemoglobin are representative of blood collected in ACD without any special precautions at the time of donation. This is a fairer reflection of the conditions which actually apply in the freezing programme and only when special precautions are taken to arrange clean venepunctures without needle transference of samples and avoidance of mechanical pressure on the syringe contents, so that rapid ejection does not occur, can one really obtain true values. A small series done in this way (Fig. 4/28) among members of Blood Transfusion Staff showed supernatant free plasma haemoglobins of 0 to 3.53 mgm/100 ml (mean 1.08 $\pm$ 2.76 mg/dl, n= 6) - a finding similar to the truly normal values shown in the Table. Tullis et al (1958) recorded the higher than normal values found in patients with various illnesses.

WHAT IS A SAFE LEVEL OF SUPERNATANT HAEMOGLOBIN?

Whole blood collected in plastic bags containing ACD solution and stored at 4°C for 21 days usually has a supernatant haemoglobin in the bound state only, in that the level of supernatant haemoglobin usually has not exceeded the haemoglobin binding capacity of the plasma (haptoglobin level). (Cassell and Chaplin 1961; Latham 1959; Laurell and Nyman 1957; Strumia, Colwell and Ellenberg 1955). If all of the supernatant haemoglobin in the units of preserved reconstituted blood exists in the bound state (the haemoglobin - haptoglobin complex) when infused, haemoglobinuria should not occur (Latham 1959; Laurell and Nyman 1957). When plasma is used as resuspension medium for the recovered cells haptoglobin is available to bind supernatant haemoglobin. The haptoglobin in normal plasma is 50 mgm to 140 mgm/100 ml with a mean of 90 mgm/100 ml (Laurell and Nyman 1957). Units of preserved red cells resuspended in autologous plasma stored at 4°C for 1-14 days show a range of total supernatant haemoglobin of 360-980 mgm on the day of transfusion by the method, and techniques of Valeri (1965). When plasma is used as a resuspension medium and assuming a haptoglobin level of 100 mgm/100 ml and a plasma volume of 250 cc approximately 250 mgm of the total supernatant haemoglobin on the day of transfusion can be bound. Therefore in a unit of preserved blood resuspended in plasma and having say 980 mgm of total supernatant haemoglobin, 250 mgm would be bound and 730 mgm would be in the free unbound state. If a recipient had a haemoglobin binding capacity of approximately 100 mgm/100 ml and a plasma volume of 3000 ml then 3000 mgm of haemoglobin or 4 units of this

blood could be transfused without haemoglobinuria occurring in the recipient. Recovered cells show a total range of 180-740mgm supernatant haemoglobin and if all of this is in free (as it would be) in an albumin or saline solution which lack any haptoglobin binding properties (Aber and Rowe 1960; Nyman 1960) then all of the supernatant must be bound otherwise haemoglobinuria may occur. It is possible to work out the maximum number of units of recovered cells which can be given intravenously before this point of full saturation of haptoglobin is reacted, provided one knows the total amount of haemoglobin supernate being infused, the size of the patient's blood plasma volume, can be assured that he is not haemolysing cells in the vascular compartment and his haptoglobin level is known or can be assumed. Renal tubular re-absorption also plays a part in determining if sufficient free haemoglobin will be excreted in the urine. (Latham 1959; Latham and Jenson 1962). From time to time reports have been written that haemoglobinuria under conditions of hypotension during surgery and anoxia may be nephrotoxic but most would agree today that the renal damage is part of the syndromes associated with prolonged renal tubular anoxia due to low rates of perfusion in the efferent arterioles and not to the haemoglobin per se (Bing 1944; Gilligan et al 1941).

Excessive amounts up to 100 times the maximum quantity of haemoglobin which would be given in the form of frozen cells were given to rabbits by Tullis et al (1958). No deleterious effect or iron accumulation was evident in post mortem tissues (Zwilling 1958). The

relative safety of such supernatant haemoglobin is probably due to its being rendered stroma free which Rabiner (1968) reviewed as being an important substance in the initiation of disseminated intravascular (incompatible) coagulation and renal failure. Stroma free haemoglobin solution was at one time used as a plasma volume expander. (Hamilton et al 1947; Phillips and Hamilton 1948; Brandt et al 1951; Rosoff and Walter 1952; Valeri 1964; Rabiner et al 1968; Rudowski et al 1968 a,b).

The haemoglobinuria with an without acute renal insufficiency noted by Valeri and Henderson (1964) following transfusion of deglycerolised cells suspended in outdated albumin was subsequently shown to be associated with albumin denaturation and not in any way due to the free haemoglobin in the supernatant (Henderson 1963 cited by Lund 1964; Tullis et al 1963).

Haemoglobinuria, after infusion of reconstituted cells, in excess of the amount of supernatant haemoglobin infused into the patients plasma volume (unexplained haemoglobinaemia) indicates either intravascular destruction of the red blood cells and/or extravascular sequestration with detectable haemoglobinaemia (Mollison 1961). Non viable red blood cells are removed within ten minutes of infusion (Strumia et al 1962; Valeri et al 1966, 1967a). If transfused frozen cells are viable in the circulation then the plasma haemoglobin concentration should not rise beyond what would be the calculated value based on the total amount of supernatant haemoglobin infused and the estimated recipients blood plasma volume. This was in fact the case found by Tullis et al (1958) and Valeri 1965, 1967 using deglycerolised cells recovered from a

-80°C bank although some plasma haemoglobin was probably being cleared even whilst the transfusion was proceeding. Cassell and Chaplin (1961) noted that the percentage of estimated rise was 57% if the blood was infused such that less than 400 mgm supernatant haemoglobin was given in less than 30 minutes. Valeri himself recorded that after infusion of 3.5 gm to 4.7 gm of supernatant haemoglobin alone to the same recipient during 40 and 45 minutes, 85% and 96% of the estimated values for plasma haemoglobin were observed as earlier described by Tullis et al (1958). The levels of supernatant haemoglobin in the final transfused product are, among other things, partly dependent on the in vitro resuspension medium. Thus autologous or heterologous compatible plasma is much superior to albumin solutions which in turn are superior to saline suspensions (p_{182}) (Tullis et al 1958; Valeri et al 1965). These same authors also noted that the in vitro storage time was correlated with the presence or absence of plasma and that even if the supernatant resuspension medium was removed prior to infusion in order to lessen the free haemoglobin load, the cells resuspended in plasma showed much better post transfusion survival. If washing is required to reduce the level of supernatant haemoglobin in units stored at 4°C for varying periods prior to transfusion than additional losses up to 10 or 15% have been reported. When it is realised that the ultimate post transfusion survival of the recovered erythrocytes may be only 70% (especially if substantial periods of pre-freeze and post thaw storage have been carried out) then the survival of the viable erythrocytes may represent a smaller proportion

of the erythrocytes originally bled from the donor. (Valeri, Fowler and Sobucki 1965). Almond and Valeri (1967) in a detailed study of patients with a wide range of diseases found no adverse effects attributable to frozen cells. If differences in packed cell volume were taken into account, patient haemoglobin and haematocrit were comparable to non-frozen cell usage. Plasma haemoglobin averaged 10.66 mgm/100 ml ± 8.84 for the frozen cell recipients and 1.57 ± 1.53 for the non-frozen cell recipients. None showed any haemoglobinuria

Perrault et al (1967) described transfusion on a patient who developed transient haemoglobinuria and whose haptoglobin levels before and after transfusion were zero. Another patient whose pre-transfusion haptoglobin level was zero did not develop any haemoglobinuria. In 23 other patients who received from 1 to 5 units of frozen cells with commensurate rises in red cell haematocrit, there was no haemoglobinuria but a moderate reduction in haptoglobin concentration was found (maximum fall of 190 mgm/dl, from 470 to 280 mgm/dl).

Valeri and Runck (1969) recorded an increase of 146 mgm haemoglobin/unit in thawed cells resuspended for 4 days at 4°C in saline when recovery was made by Huggins cytoagglomeration method. Continuous centrifugation methods of recovery gave extremely high levels of haemoglobin in the supernate. High glycerol slow freeze processed red cells stored frozen without EDTA up to 8 months can be kept for up to 6 days at 4°C in a saline medium with acceptable post transfusion survival (Valeri and Bond 1966). Similarly processed cells stored for up to 2 years at -80°C

could be stored at 4°C for approximately 24 hours, while storage of these red cells at -80°C for up to 3 years permitted maintenance in the post thaw state for only about 4 hours. Albumin was found not to improve the post thaw stability. With Cohn programmed centrifugal washing these authors were eventually able to obtain good in vivo survival after post thaw resuspension for 24 hours at 4°C provided the glycerol on the cells underwent a predilutional step before washing. The use of EDTA in the Huggins procedure adversely affects both the recovery in vitro and the survival in vivo while it prevents the complement coating of the red blood cells exposed to low ionic environment. At that time Valeri and Runck (1969) stated their case rather succinctly: "if 250 ml of red blood cells are frozen within 1 day of collection, stored for 6 years, thawed washed and resuspended and stored for 24 hours at 4°C the stable anaemic recipient will have received 150 ml of red blood cells that will be viable 24 hours following transfusion and thereafter have normal long term transfusion survival". Total supernatant haemoglobin is approximately 450 mgm/unit for Huggins method and 525 mgm/unit for Cohn method (see p.191,supernatant haemoglobin).

Later the same year Valeri and Runck (1969b) investigated various ways of deglycerolising cells stored in liquid nitrogen by the low glycerol rapid freeze technique. Average supernatant haemoglobin in recovered washed and resuspended units showed a range from 102 to 699 mgm total haemoglobin in the supernate of units (mean 457 ± 158). After storage at 4°C for up to 72 hours

the supernatant haemoglobin per unit ranged from 360 to 1728 mgm (mean 973 ± 445 mgm) (p.186,post thaw haemolysis on storage at 4°C). The smaller the freeze thaw haemolysis, the lower the total amount of supernatant haemoglobin in the unit following washing. Units with the least haemolysis survived best, and similarly units with least supernatant haemoglobin on the day of infusion had best survival values. Intracellular potassium values paralleled their findings i.e. the higher the cellular potassium on the day of infusion, the better the 24 hour survival. All of this of course, is merely a reflection on the amount of cells which have escaped irreversible injury during the whole procedures of glycerolisation, freezing, thawing and resuspension and are what one might expect having regard to earlier observations (p.33). Similarly the recipient's plasma haemoglobin values immediately post transfusion ranged from 0.6 mgm to 3.0 mgm/100 ml which were consistent with expected values and indicated levels calculated on the supernatant haemoglobin in the transfusion and not any fresh haemolysis occurring in vivo indicating the success of this method in minimizing freeze thaw injury to cells. Expected plasma haemoglobin after transfusion is calculated as

$$\frac{\text{mgm infused supernatant haemoglobin}}{\text{ml recipients' plasma volume}} \times 10$$

In a later set of observations Valeri et al (1970) presented further evidence that (a) EDTA adversely affects red cell membranes and results in greater susceptibility to freeze thaw injury and (b) EDTA enhances poor cell recovery

when combined with the Huggins cytoagglomeration method of red cell washing (c) such cells could only be stored a few hours before transfusion otherwise further deterioration would occur. Better results were obtained using continuous centrifuge washing in electrolyte solutions. The low ionic environment of the agglomeration produced a significant reduction in intracellular potassium, poor 24 hour post transfusion survival, decreased recovery of cells in vitro and a higher supernatant haemoglobin concentration. In addition there is evidence that additional intravascular destruction of compatible non-viable cells occurred in two patients out of seven. Similar results of intravascular destruction of non-viable cells recovered and processed by Huggins method have been reported by Kamegai and Sumida (1968) and Sumida and Toshihiko (1968).

When discussing the influence of haptoglobin binding on free and bound plasma haemoglobin levels it is as well to remember the variations which occur with age from those stated above which apply to adults. Thus, Lundh, Oski and Gardner (1970), found the level to be 13 mgm/100 ml for full term infants, (range 0-70); 3 mgm/100 ml for premature babies (range 0-26).

Nyman (1954) found in a group of 150 blood donors an average of 109 $\pm$ 40 mgm haptoglobin/100 ml. De Castro et al (1966) in a group of overbled donors found elevated values of 216 $\pm$ 83 mgm/10 ml in contrast to a group of non-donors who showed levels of 115 $\pm$ 27 mgm/100 ml. Langley et al (1962) showed that serum haptoglobin level decreases after the intravenous injection of haemoglobin

or after acute increase in red blood cell destruction. Laurell and Gronwall (1962) believe that in adult man about 1.4 gm of haptoglobin are consumed daily by the 0.5 gm of haemoglobin normally released into the blood stream. This represents about 20% of the total pool of 8 gm of haptoglobin, another 10-20% being destroyed by normal catabolism daily. In other words about half of the daily turnover of haptoglobin would be consumed by the haemoglobin released into the blood stream. Adult female haptoglobin is usually about 20 mgm/100 ml lower than in adult males.

In investigating the effects of prior anticoagulation of donor blood with either ACD or CPD, Valeri (1974) showed that blood stored for 4 days prefreezing had a supernatant haemoglobin of 543 $\pm$ 501 mgm/unit and for CPD storage the level was 603 $\pm$ 310 mgm/unit transfused. No haemoglobinaemia was associated with the removal of non-viable but compatible red cells and if anything the observed level of plasma haemoglobin was lower than the theoretical value estimated from the supernatant infused and the recipients plasma volume using the formula quoted above.

Akerblom and Hogman (1974) investigating rapid freeze low glycerol method in cryogenically resistant bags, made of Teflon, with various forms of recovery commented on the much lower recoveries of cells and higher supernatant haemoglobin values in some cases. There could be enough free haemoglobin to exceed the haemoglobin binding capacity of haptoglobin if more than 2 or 3 units of agglomerated cells were given. This could result in haemoglobinuria. However as noted by Huggins (1966) a single centrifugation

and removal of supernatant fluid before transfusion practically eliminates this risk. Doing this of course, is really combining two methods - cytoagglomeration and batch centrifugation.

These observations on supernatant haemoglobin, its supposed toxicity and means of removal have been described in detail here in order to permit a careful appraisal of the clinical data now to be described.

STUDIES ON 2,3 DIPHOSPHOGLYCERATE

It has been shown by numerous workers (Mitchell 1975 for review) that the level of 2,3 diphosphoglycerate (2,3 DPG) in red cells is crucial to their oxygen affinity. In order to investigate the effects of storage before and after freezing and thawing a study was made using commercially available kit for such estimations (Sigma Chemical Co.). The outlines of the Principle of this test are given in the technical appendix.

Donor blood was collected by a standard technique into ACD anticoagulant and stored at 4°C for varying intervals before testing. Later it was possible to test a batch of similar donations taken into CPD to determine if the anticoagulant would substantially modify the outcome of the red cell haemoglobin affinity for oxygen. In another experiment blood was taken and frozen by the method of low glycerol rapid freeze outlined in the technical appendix. Thawing and recovery by batch washing was carried out after varying times in liquid nitrogen storage up to 1 year and assays of 2,3 DPG made at the time of final preparative washing in the batch method, unless otherwise stated the red cells from the normal donors were 2 days old when tested and the thawed recovered cells were from units frozen down at 2 days post donation. Some units were studied over a longer or shorter period of time in order to assay the 2,3 DPG levels in ACD blood with longer shelf life.

In some units of CPD blood assays were performed from day 1 to day 29 to assess the long shelf life of CPD anticoagulated blood. In this latter respect the final

study of CPD anticoagulated frozen blood is not complete since the main part of this study was performed when the regular routine anticoagulant used in all donations collected was ACD.

Table 4/29 shows the mean and standard deviation for 2,3 DPG assay in ACD and CPD blood before freezing. Both sets of donations were stored at 4°C for 2 days before testing.

Table 4/29. 2,3 DPG with ACD and CPD frozen blood.

Number of donations	µ moles/ml whole blood	µ moles/ml packed cells	µ moles/gram haemoglobin
33 ACD	1.2374±0.3098	1.9892±0.6229	6.6119±6.307
38 CPD	1.4444±0.2555	2.9336±0.7267	9.4302±6.988
(2 days old)	t test:-	0.001>P	0.001>P

Blood 2 days old was chosen for the tests since most units included in the frozen bank are less than 5 days old on the day of freezing in common with most other workers.

Analysis of the differences, if any, between these two series of ACD and CPD blood have shown that there is a statistical difference and that blood collected in ACD is not quite as good as blood in CPD for freezing.

Assays of ACD blood (at 2 days old) before and after freezing and thawing have shown the results in Table 4/30:-

Table 4/30. 2,3 DPG with ACD before and after freezing.

Number of donations	µ moles/ml whole blood Col. 1	µ moles/ml packed cells Col. 2	µ moles/gram haemoglobin Col. 3
Pre-freeze ACD (33)	1.2374±0.3098	1.9892±0.6229	6.6119±6.3071
Post thaw ACD (32)	1.3428±0.1562	2.3650±0.5158	7.4812±5.3301
t test:-	0.4>P>0.3	0.05>P>0.025	0.2>P>0.1

Analysis of these results by comparison of the mean and standard deviations has shown that there is no significant difference between the 2,3 DPG levels in cells before freezing and on thaw recovery prior to infusion into patients. This is

a most encouraging finding since it establishes that the red cells do not deteriorate in frozen storage for prolonged periods of time. This is in contrast to the findings shown in Table which outlines the steady decline in 2,3 DPG observed with conventionally stored blood in CPD.

Table 4/31. 2,3 DPG decline in liquid CPD blood.

CPD Stored blood at 4°C for	µ moles/ml whole blood	µ moles/ml packed cells	µ moles/gram haemoglobin	No. of dpnations averaged
Day 1	(1.4800)	3.556	10.6616	6
2	1.327	2.952	8.7466	67
3	1.048	2.870	8.7533	6
4	0.8466	4.1333	9.0400	3
5	1.63	3.89	8.87	1
6	0.760	2.013	6.843	4
7	1.046	2.185	6.633	6
8	1.1975	2.5425	7.5075	4
9	0.8850	1.8783	6.5266	6
10	0.7080	1.4360	4.3444	5
11	1.1500	2.4500	7.3866	3
12	0.74	1.45	4.20	1
13	0.76	1.41	4.14	1
14	6.6066	1.1766	3.4800	3
15	0.4065	0.800	2.503	3
16	0.2125	0.465	1.4025	4
17	0.200	0.405	1.220	4
18	0.1523	0.3133	0.95	3
19	0.13	0.35	1.090	1
20	0.07	0.14	0.44	1
21	0.1035	0.1975	0.62	4
22	0.064	0.1385	0.415	2
23	0.09	0.22	0.69	1
24	0.05	0.13	0.40	1
[illegible]	0.07	0.16	0.485	2
	0.07	0.17	0.50	1

Since frozen cells recovered from liquid nitrogen are usually given a shelf life of 12 hours because of the bacteriological hazard of opportunistic inoculation of micro-

organisms some recovered cells were kept deliberately beyond this natural life in order to assay the effect on the cell 2,3 DPG levels, in one case up to 72 hours post thaw and in another up to 11 days post thaw.

Results are shown in the Table

Table 4/32. 2,3 DPG in thawed blood (ACD).

Donor No. & days post thaw	μ moles/ml whole blood	μ moles/ml packed cells	μ moles/gram haemoglobin
76818 0	0.26	0.48	1.61
1	0.26	0.52	1.68
2	0.15	0.25	0.80
3	0.14	0.26	0.87
92915 0	1.13	2.14	6.15
1	0.90	1.70	4.90
2	0.62	1.18	3.40
3	0.36	0.68	2.20
4	0.21	0.38	1.12
7	0.10	0.19	0.54
8	0.08	0.16	0.45
10	0.09	0.17	0.50
11	0.05	0.09	0.25

These studies show that if a graph is prepared to combine the tables for the decline of 2,3 DPG blood in normal non-frozen units with the decline in previously frozen recovered units the rate of decline is very similar (Fig 4/32) illustrating that frozen cells upon recovery behave in an analogous way to non-frozen conventionally stored blood.

Few studies of this nature have been revealed on a search of the literature. Such as these are presented for comparison in the Table 4/33.

From this scanty information from the literature it is confirmed that 2,3 DPG levels are maintained near pre-freeze levels so that the cells prepared by the method chosen (low glycerol rapid freeze) is adequate to maintain the red

Author	Post thaw cells (μ mol/ml cell)	No. of donors
Prins and Loos (1970)		
4°C 1 day	0.48±0.05	10
4°C 8 days	0.13±0.04	9
4°C 15 days	0.02±0.02	10
4°C 21 days	0.0 ±0.01	8
Akerblom and Hogman (1970) 4°C 2-6 days	22-36 μ mol/10^{12} RBC pre-freeze	4
Centrifuge wash	28-37 μ mol/10^{12} RBC centrifuge wash	4
Agglomeration wash	35-38 agglomeration wash	4

Table 4/33. 2,3 DPG in frozen blood.

cells in as near physiological normality as possible. It is likely that these cells will behave quickly and efficiently in the circulation of a recipient and would be of considerable help in the hypoxic patient and in those whose condition may be worsened by infusion of a non-physiological banked blood which can do no more than replace circulatory blood volume and may require a period of some hours in vivo before full restoration of the depleted 2,3 DPG stores. (review, Mitchell 1976).

Valeri (1974) reported the restoration in vivo of 2,3 DPG, ATP, Potassium and Sodium ion concentrations of non frozen blood stored up to 15 days at 4°C. The 2,3 DPG level rose by 25% in 8 hours and 50% in 24 hours towards the pre-storage value. Because of this slow rise in 2,3 DPG in patients incapable of increasing their coronary flow, congestive cardiac failure may result from infusions of large amounts of 2,3 DPG depleted blood. Frozen red cells, since they are usually stored when only a few days old, do therefore maintain their 2,3 DPG levels and may maintain better function. The CPD and ACD blood 2,3 DPG levels before freezing are significantly in favour of CPD. Valeri (1974) reported that CPD anticoagulated blood maintained the oxygen transport much better than ACD during storage of red cells at 4°C for up to 1 week prior to freezing.

During the preparation and process recovery of frozen cells studies were made on the white cell counts before glycerolization, after glycerolization and after freezing, thawing and batch washing. White counts were done on automated particle counting equipment (Coulter Electronics). As a check on this, blood films which had been prepared for morphological study were used to identify any white cells. The white counts at various stages of preparation were:- Table 4/34

Average	ACD pre glycerol	After glycerol	Post Thaw washing	Total Units examined
WBC/cumm	4450	3250	333.6	97

The post thaw counts are almost certainly in error since within the sample material examined remnants of leucocyte and other debris are present (see p 169). Certainly in the blood films examined, very very few leucocytes are present and one has to search diligently to find any recognisable cells. Even these are damaged and often show loss of cell detail with karyolysis and poor staining properties. These figures again show the generally held view that the use of frozen cells is the best way of removing leucocytes viable, non-viable, fragmented or lysed (see Removal of Leucocytes). No platelets were noted in any blood film.

AMORPHOUS DEBRIS SEEN AT RECOVERY

Before leaving the quality suspension media and their plasma proteins the results of a study on the gelatinous amorphous material left in the transfusion bag and giving set filters will be given. This material is regularly present during the recovery of cells and forms gelatineus masses of a stringy consistency clinging to the wall of the bag. Most of it can be removed with the wash solution decant provided care is taken to coax it into the transfer line whereupon it usually slides along quite freely. It is only recently that the true nature of this substance has been elucidated (Valeri 1974) but our earlier results are still valid. It was collected from bags by saline wash out and concentrated by centrifugation. It is insoluble in warm or cold saline but easily hydrolysed by N/1 sodium hydroxide and is soluble in 5M urea. In chloroform water it forms an obvious protein band on centrifugation with some chloroform solubility. The protein suspension and chloroform extract were examined by electrophoresis on cellulose acetate strip and shown to be protein with no lipid present when stained by lipid and protein stains. Immunodiffusion study of the protein suspension against anti-fibrin, anti-human globulin, anti-IgG, anti-IgA, anti-IgM of rabbit origin showed no evidence of any fibrinogen or fibrin degradation product. The only line of identity was against the anti-human globulin serum. Light microscopy of material fixed in 10% formation and stained by the Feulgen method showed numerous white cells closely packed together in various stages of destruction. Few red cells were present. It has recently been shown (Valeri 1972) that this material is probably of leucocyte and

platelet origins. It is rich in nuclear deoxyribonucleic acid (DNA), and can be removed during processing by addition of the enzyme DNAase to the wash solutions.

SODIUM STUDIES

Since the sodium ion is intricately involved in the ionic fluxes which occur across cell membrane under the action of ATP and since it constitutes an important ion in determining cell hydration, various authors have studied it whilst involved in potassium observations. Techniques are simple and can be done in parallel with potassium studies using the flame photometer (Table 4/35).

Rowe et al (1970, 1971) showed that there is approximately a 10% increase in cell sodium indicating some slight deterioration in cell permeability but this is rapidly restored after recovery when cells were incubated at 37°C in ACD plasma. Serial studies at 7, 14 and 21 days storage at 4°C before freezing and post thaw showed no significant difference and anti-coagulation with ACD or CPD had no obvious effect.

Supernatant sodium levels have for the reasons given above also been measured (Table 4/35).

The findings in the present study show, if anything, a slight reduction in the supernatant sodium in the post thaw phase. This would be in keeping with the increase in cellular sodium in recovered red cells noted above.

Since sodium ion is unusually estimated together with the chloride ion, results were collected for these same units as are shown in the table. Only one paper can be found in the literature where specific reference is made to chloride ion concentration (Huggins 1965, 1970; Huggins & Grove Rasmussen 1971) where supernatant chloride is given as 85.2 meq/litre ± 9.3. The present study showed pre-freeze levels of 76.52 ± 4.96 meq/litre (169 observations). 79.57 ± 3.89 on the glycerolised specimens (155) prior to

Author		meq/Kg cells pre-freeze	post thaw	
Derrick et al (1969)	ACD	27.3±3.2	23.8±4.1	
		33.1±4.6	25.4±6.3	
Rowe et al (1970,1971)		12.6	16.4	
Derrick et al (1972)	ACD	20.5	16.4	
		meq/litre pre-freeze	post thaw	
Huggins and Grove Rasmussen (1965) (1971)			84.3±11.6	
Perrault et al (1967) (Huggins)	ACD		85.0-95.0	
Huggins (1970)				
Orlina et al (1972)	a	132.5	119.4	
	b	137.8	124.8	
	c	131.1	129.5	
Tullis et al	ACD	96.0	105.0	
Present work	ACD	159.12±3.73	142.0	glycerolised 111.40±4.83
		(169 observations)	(157 observations)	
				157 observat

Table 4/35. Supernatant sodium content of frozen cells

freezing and 78.0 post thaw (155 specimens).

It is to be noted also that the levels of sodium and chloride mostly come from the added sodium chloride in the wash and suspension fluids and that the pre-freeze levels are elevated due to prior addition of sodium citrate as anticoagulant, thus

Disodium hydrogen citrate $Na_2 H C_6 H_5 O_7$ 1.5 H_2O mol. wt. 263.09

2.2g citrate added to 500 ml blood =

$\frac{22.99 \times 2}{263.09}$ gm sodium ion added to 500 ml blood

= 174.9 mgm sodium ion added to 500 ml blood

equivalent weight of sodium = 23 gm/1

mequivalent weight of sodium = 23 mgm/1

$\therefore$ meq/l sodium ion added to blood = $\frac{174.9 \times 2}{23} = \underline{15.2}$

POTASSIUM STUDIES

Red cells lose potassium during conventional storage in ACD or CPD and this accumulates in the suspension medium (Rowe et al 1970; Button et al 1972). A number of authors have measured cellular potassium at varying intervals before and after freeze and thawing red cells. (Table 4/36). The extracellular phase of blood during liquid nitrogen contains an excess of free potassium. At standard refrigerator temperature there is insufficient metabolic activity to maintain the high intracellular gradient with a resulting leakage of potassium into the surrounding medium. This (in ACD) averages 12 meq/l after 1 week, 17 meq/l after 2 weeks and over 20 meq/l after 3 weeks at 4^{o}C (Gibson et al 1956). When these cells are rewarmed most of the potassium is taken back inside the cells but there may be sufficient to cause hyper-kalaemia in a recipient especially in uraemia, renal failure, extensive burns and other conditions where hyper-kalaemia may be so severe that it precludes much needed transfusion therapy. Washing cells and resuspension of the thawed deglycerolised unit in potassium free media can create a "potassium sponge". Tullis and Lionetti (1966) described transfusion of 4 units of such cells into a severely hyper-kalaemic patient and recorded a fall in serum potassium from 7.82 to 6.82 meq/l with concurrent electrocardiographic improvement. They considered that owing to the anaemia which constitutes such a common complication of renal disease, this observation might be exploited in the future.

Author	meq K^+ /Kg cells pre-freeze	Post thaw
Derrick et al (1969)	61.6 ± 1.7	-
	59.0 ± 2.4	66.9 ± 3.8
	59.0 ± 2.4	68.1 ± 3.8
Krijnen (1970)	"Satisfactory"	
Valeri (1970)	"Satisfactory with no reduction"	
Rowe et al (1971)	86.3	86.5
Button et al (1972) ACD	83.0	-
Derrick et al (1972) ACD	75.3	74.4
Funder et al (1966).	90.1	

Table 4/36. Potassium content of Frozen Blood.

Rowe et al (1971) as well as showing that the intracellular potassium was unimpaired and remained at pre-freeze levels had earlier shown (1968) that this equally applied to cells which had been depleted of potassium by conventional storage at 4°C for up to 14 days before freezing. Pert, Schork and Lundberg (1968) have shown that 14 day old cells frozen and recovered could lose up to 20% potassium and cautioned against the problem of inducing hypokalaemia if such "potassium sponges" were infused in large quantity. Similarly Runck, Valeri and Sampson (1968, 1969) have shown that a non-electrolyte hypertonic washing of frozen cells by centrifugal washing (Cohn fractionator) in the high glycerol slow freeze (-80°C) method of Huggins can result in significant losses of potassium and ATP from the cells although these deficits are rapidly restored in the circulation as "power" in the form of high energy phosphate and membrane stability is restored to the cells.

As well as measuring potassium in cells in terms of meq/kgm of cells other authors have measured the ion in terms of meq/10^{12} RBC (Table 4/37).

These results although in different units of expression are nevertheless indicative of the general trend that potassium levels are preserved in all of the methods used at or about the pre-freeze level with perhaps some doubt about the Huggins low ionic hypertonic continuous flow centrifugal wash system. Indeed Almond and Valeri (1967) noted a significant decrease in serum potassium concentration in recipients of deglycerolised cells from such a system. Recovered cells had approximately

Author		pre-freeze meq/10^{12} rbc	post thaw
Valeri and Runck 1969 a		8.72 ± 0.98	6.13 ± 0.70
		8.55	7.26
Valeri and Runck 1969 b		7.40 ± 0.46	7.17 ± 0.65 after 72 hr in glucose/saline at 4°C
Tullis et al 1971 ACD		79.0	85.0 (meq/l)
Valeri 1974	1	8.64 ± 0.75	8.67 ± 0.23
	2	7.12 ± 0.84	
	3	7.89 ± 1.16	7.91 ± 1.52
	4	6.31 ± 1.94	-

Table 4/37. Potassium content of red cells.

one half of the normal intracellular potassium content and showed that on infusion the potassium depleted cells absorb potassium from the recipients plasma. These results further strengthen the case for freezing blood soon after donation and not waiting until potassium depletion has reached a low level. It has been suggested recently (Mitchell 1976 review) that older blood could be rejuvenated with metabolic additives which would restore energy metabolism and ionic balance to the cells prior to freezing as packed cells from which the otherwise toxic supernatant resuspension rejuvenating fluid has been removed but this seems unduly cumbersome (Valeri 1975).

If the potassium from damaged cells leaks into the supernatant then it might be easier as a test of red cells viability and integrity to measure this. This is technically much easier and can be carried out on bulk samples including supernatant resuspension medium at intervals with an assurance of adequate samples which can be processed in a standard flame photometer. (Table 4/38).

These findings confirm the point made earlier that there would seem to be no undue loss of potassium from the cells into the resuspension medium. Since the information in the literature is so scanty a large consecutive series of supernatant potassium levels were assayed in cells supernatant plasma before freezing (usually at 2-5 days) and immediately after thawing washing and resuspension (Table 4/38. These results concern ACD stored donations but more recently similar results have been obtained with CPD anticoagulated donations.

Author		Supernatant K⁺ meq/l Pre-freeze	post thaw
Huggins and Grove-Rasmussen (1965 1971)	ACD	-	1.9 ± 0.62
Prins and Loos (1965)		4.0	-
Perrault et al (1967) (Huggins)	ACD		3.05 (0.9-5.4)
Daszynski and Maj (1968)		4.3 ± 0.9 (3.25-5.6)	5.3 ± 0.7 (4.52-6.77)
Shields (1968)	ACD	4.2	-
Huggins (1970)	ACD		
Tullis et al (1971)	ACD	3.6	3.4
Button et al (1972)	ACD	7.0	-
Gibson et al (1972)	ACD	5.5	4.7
	CPD	3.7	2.6
Orlina (1972)	a	9.26	20.29
	b	10.03	19.7
	c	8.51	15.8
Present Work (1972)		6.81 ± 1.33 (165 observations)	0.76 (157 observations)
Valeri (1974)	1	3.5 ± 1.1	4.4 ± 2.4
	2	1.0 ± 0.6	4.0 ± 3.6
	3	2.7 ± 0.8	5.4 ± 2.3
	4	3.7 ± 2.0	3.8 ± 0.9
		glycerolised blood 4.3 ± 1.40 (157 observations)	

Table 4/38. Supernatant potassium on frozen cells.

Studies on Hydrogen Ion Concentration

Changes in hydrogen ion concentration (pH) have an influence on red cell metabolism and can modify the ability of red cells to carry oxygen (Review Mitchell 1976). Table 4/39 shows the recorded pH values in the literature together with a much larger series done by the author on consecutive units using a standard glass electrode pH meter.

The results show that on recovery, frozen red cells do not substantially alter pH and the extensive work of Valeri (1974) certainly shows that it is possible to maintain pH at post thaw levels without any significant change. Dawson et al (1972) showed that hydrogen ion concentrations in the suspension medium of red cells in excess of 1×10^{-7} (pH 7.0) led to a rapid fall in red cell 2,3 DPG levels and therefore loss of haemoglobin function. They recommended that preservatives should have a pH of greater than 5.5 to maintain optimal conditions for haemoglobin function and red cell viability (ATP levels) although some additional adenine may be required. Haynes et al (1960) recorded that too acid pH resulted in excessive haemolysis. The optimum was pH 7.0 which gave supernatant haemoglobins after processing of 35-200 mgm/dl.

Studies on Enzymes (Chilson et al 1965).

Few workers have measured the enzyme activity in processed cells. Krijnen et al (1964) and Prins et al (1965) reported no significant differences between frozen and non-frozen cells content of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pyruvate kinase and glutathione reductase. Enolase, 3

Author		Pre-freeze		Recovered Cells	
Tullis et al (1958)				7.1	
O'Brien and Watkins (1960) ACD normal		7.10 at 4°C		maximal survival in vivo	
Rowe et al (1968) ACD normal		6.60		-	
Shields (1968) ACD normal		7.38		-	
Perrault et al (1967) ACD Huggins				6.96 (6.65–7.28) (n= 12)	
Derrick et al (1969)		6.65 ± 0.60 6.64 ± 0.10		6.25 ± 0.30 6.24 ± 0.30	
Gibson et al (1972) ACD		6.84		6.98	
		7.06		series 1 7.22 (= 20) series 2 7.13 ± 0.10 (n= 9	
Orlina et al (1972)	a	6.039		5.90	
	b	6.73		6.68	
	c	6.84		6.85	
Valeri (1974) ACD	1	-	6.73 ± 0.11	6.72 ± 0.14	
	2	-	6.77 ± 0.11	6.80 ± 0.17	after 24 hr
	3	-	6.85 ± 0.22	6.78 ± 0.06	
		-	6.71 ± 0.04	6.77 ± 0.14	
CPD	1	-	6.91 ± 0.09	6.97 ± 0.04	after 24 hr
	2	-	6.93 ± 0.08	7.03 ± 0.05	
	3	-	6.93 ± 0.10	6.87 ± 0.06	
	4	-	6.87 ± 0.20	-	
Present author		7.04		7.09 n = 120	

0–4 hr

0–4 hr

Table 4/39. Hydrogen ion concentration

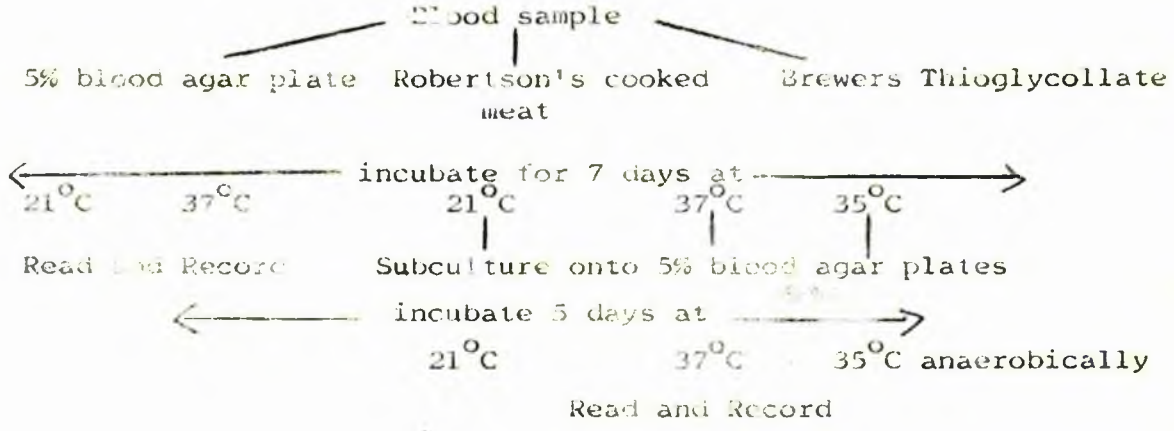
phosphoglycerate kinase, pyruvate kinase, adenosine triphosphatase, nucleoside diphospho kinase, glutathione reductase and lactate dehydrogenase remained unchanged in blood stored at -120°C (Mourad 1969). Enzyme dependent transport systems for sodium and potassium appear to be unimpaired in cryopreserved cells (Pert et al 1967; Derrick et al 1969). Valeri et al (1966b) reported that in vivo survival of red cells recovered by Huggins techniques was correlated with the enzymes of glycolysis, hexokinase, glucose 6-phosphate dehydrogenase, glutathione reductase and glutathione stability. Using small sample freezing by droplets sprayed into liquid nitrogen (see p66) Rowe et al (1967), Seidle et al (1972) show that the activity of glucose-6-phosphate dehydrogenase, lactic dehydrogenase, glutamic pyruvate transaminase, glutamic oxalacetic transaminase and aldolase were unimpaired on recovery. Metabolically frozen recovered cells behave as if they never had been frozen (Tullis and Lionetti 1966) with retention of glycolytic function and oxygen carrying capacity and normal oxygen exchange functions (Sendroy and O'Neal 1960; O'Brien and Watkins 1960).

Bacteriological Studies

One of the problems of any system employing multiple transfer of reagents is the maintenance of sterility. Blood products are especially vulnerable to this since in the processing multiple opportunities exist for ingress and growth of micro-organisms. When it was decided to start a frozen cell bank it was clear that all units of frozen blood must be treated like any other

blood product and subjected to stringent bacteriological surveillance. Since each unit is individually prepared then each unit must be individually examined and reported upon. This is different, for example, from the usual sampling by random spot checks on plasma for drying. I was fortunate in this respect in that the West of Scotland Blood Transfusion Centre has always had a heavy commitment in drying plasma for Scotland and Northern Ireland. For this a good department of bacteriology exists with expert technical assistance. The results of the testing of every unit of frozen cells are noted on the donor's record card which was designed by me (**Vol 2.**). As experience and confidence in the product grew there was naturally a desire to reduce the amount of quality control work on each unit. Most tests were discontinued after 1 year's full operation except the bacteriology tests which are assiduously followed.

The schedule followed is shown in the figure. 10-15 ml samples of blood are taken at the end of the washing recovery process through the last of the transfer lines of the plastic washing bag (see p 74).



All units of blood from the frozen bank have been sterile. Since it can be argued that a blood sample taken from a bag and then cultured might not be entirely representative of the entire unit and that a sample taken at the moment of issue when the shelf life is actually 12 hours it was decided to prepare units which closely resembled the actual shelf life conditions. If the shelf life has to be extended then it would be useful to know that sterility could be maintained for longer than 12 hours. Eight units of processed blood which had been prepared as for patients were kept in the blood bank refrigerator at 4°C for up to 10 days. A Fenwal multiple injection adaptor (AE 9) was inserted and left in the giving port of the blood bag and samples of the thoroughly mixed contents withdrawn everyday for the period of the experiment. All of these samples remained sterile.

All of the preparation fluids, the glycerol, sorbitol wash and sodium chloride are also tested by the same rigorous quality control before being issued for use. In addition pyrogen testing to B.P. standards is done on the wash solutions which are all prepared at the Regional Transfusion Centre.

The author has only heard of one case of septicaemia due to infected frozen recovered cells in another U.K. centre. The patient, a young woman, on regular haemodialysis received cells which had been thawed in a water bath which contained water later shown to have a growth of pyocyaneus which caused the patient to collapse soon after receiving the cells. The same organism was recovered from the patient's blood and the washings and

residue of the cells in the plastic bag. It is therefore important to avoid splashing of water during the thawing and to ensure that before the thawed cells are removed to the washing bag that all puncture sites are swabbed with phenol-glycerine.

Virological testing of donations is also important and this has been dealt with on p88 . No experiments have been done on the presence of viral antigens in frozen cells by the author although a few units of blood known to be viraemic are in store. Numerous authors have reported that all units of frozen cells in their systems have remained sterile after culture on blood agar, thioglycollate and Sabouraud's medium following deglycerolisation. None have discussed the prolonged culture of cells at intervals beyond the recognised storage time although some have advocated this approach to extend the shelf life beyond 12 hours. Not every author has recorded the bacteriological findings although one presumes that they have carried out these tests. (Tullis et al 1958; Haynes et al 1960; Huggins 1965; Valeri and Bond 1966; Valeri et al 1966; 1967; Perrault 1967; Valeri and Runck 1969 a,b; Valeri et al 1970 a,b; Valeri, Szymanski and Runck 1970; Tullis et al 1971; Gamshirt and Seidl 1974).

Because of the batch washing anti-A or anti-B were not detected when the final product was examined. This is not unexpected and confirms the experience of all other workers who consider that frozen cells of group O are, by virtue of their freedom from alloantibodies, much more akin to the concept of universal donor (see p. 149).

To further test the efficiency of the processing to remove alloantibodies immulogical methods were used to study the removal of plasma proteins in the wash solutions. Using a rabbit anti-human globulin serum as the antibody and cell washings as the source of possible antigen, immunodiffusion tests were carried out in a standard Ouchterolony plate. No human plasma protein other than free supernatant haemoglobin was found in the final washings. No abnormal antibodies have ever been found in the resuspension medium and red cells from antibody carrying donors form a good source of red cells for freezing after separation of antibody from the donation.

At the time of starting this project in 1971 a few reports were available in the literature on the retention of full red cell antigenicity after freezing and thawing. Several methods had been introduced for the storage of small aliquots for panels of antibody identifying cells. (see p 33). In all of the methods reported for use with frozen bulk storage the erythrocytes showed no alternation in antigenicity (Huntsman et al 1965). Only one minor problem has been described that the direct antiglobulin on the recovered cells is often positive due to a coating of components of complement (Valeri and Runck 1969) when cells are recovered by the low ionic strength cytoagglomeration technique of Huggins. Nevertheless these cells show normal survival when transfused. Very recently Moore et al (1974) reported the interesting finding of four donors whose cells during the freezing process acquired a coating of complement components C_4^b and C_3d due to previously undetected cold agglutinins in these donors' plasma. So far we have not had any difficulty with recovered cells and their antigenicity for a wide range of antigens has been maintained at least for the 3-4 years of our study. This would be the experience of other frozen cell users and Huggins has reported the use of frozen cells after 10 years storage. Since <u>every</u> time cells are recovered a matching test is performed against the patient's serum there is a continuous check on the reactivity of the red cells, now approaching 3000 donations.

Cells from our bank have been assessed at various intervals (Mitchell 1972). Rowe et al (1967) recorded that 70 different human blood group antigens remained intact

after freezing and thawing. They did not find any "abnormal" antibodies resulting from the use of frozen cells although they did state that "normal" antibodies could be stimulated and produced in primates. Unfortunately no other details are given.

The author did produce an anti-N and an anti-M in rabbits using frozen cells without any difficulty.

To date no patient who has received frozen cells from our banks has developed any new antibodies. In most cases blood is selected for patients purely on the requirement rhesus negative or rhesus positive group O. This is not unexpected since if ABO and rhesus homologous blood is chosen for patients there is much less chance of transfusing incompatible cells (Mollison 1974).

In view of the medicolegal implications of the choice of cells for production of anti-D in human volunteers, Cook (1975) decided to use group O frozen cells in a group of male volunteers in Inverness. In our Centre a small pilot study along similar lines was started in female volunteers who had already been immunised by pregnancy and had become infertile by tubal ligation, hysterectomy or menopause. Both of these studies are not yet complete but both have succeeded in producing additional anti-D plasma by plasmaphoresis for the Scottish Blood Products Fractionation Centre. It is unfortunate that the use of frozen cells as immunogens in primates (monkeys) by Rowe et al (1967) already referred to has no details of the injection immunisation schedule. The higher primate (human) would seem to vary considerably in the ability to respond to frozen cells. Cook (1975) has with our help tried three different schedules by (a) varying the dose of cells used for the induction and (b) varying the time

interval between subsequent doses. Because some donor units are fully blood grouped (see Vol 2) it is possible knowing the volunteers own blood group profile to select from the frozen inventory files donors who are antigenically identical except in respect of their rhesus antigens. In this way one should produce only the desirable rhesus antibodies and not develop any other antibody which if repeated in other systems due to multiple boosting could render the volunteer untransfusable or at least make the selection of compatible blood of unusual complexity. This would be accentuated if the volunteer required transfusion in an emergency or were hospitalised in a country where technical facilities might not be readily available or very costly. For this reason Cook is probably correct and when one considers the other features of frozen cells as freedom from platelets or white cells and freedom from virus transmission the argument is even stronger. Donors who are used for boosting volunteers have their cells kept in liquid nitrogen for at least 4-6 months so that the incubation time for virus hepatitis will pass and the donor report sick if he developed any clinical upset. This, of course, does not preclude the equally important (some would say more important) donor who has a subclinical infection and is in the prodromal phase of a subclinical attack at the time of donation. No test yet developed can detect all of these individuals but they are likely to be rare combinations of events - the prodromal non-viraemic undetectable person who donates blood at that precise moment. Nevertheless all donations in the frozen cell bank must be tested for hepatitis virus and this has always been our policy. As newer and more sensitive tests

have been developed and became available frozen blood has been cleared for use in common with all other donations in the West of Scotland Transfusion Centre. In this way red cells of "known pedigree" can be "accredited" for use with the volunteer panel for antibody production.

In the Inverness series, 10 men have been given frozen cells. Five have made and continue to make anti-D ranging in titre from 0 to 60,000 by IAGT, representing a microgram antibody content of 0.0/ml to 630/ml. A second series of men has now been started on a similar programme. Results will be reported elsewhere (Cook and Mitchell of anti-D).

Regular boosting has been used to maintain or raise anti-D levels. Volunteers are usually retired after many months as new individuals are recruited.

There are as many schedules for raising anti-D as there are investigators and a meeting was held under the auspices of the British Society for Immunology in October 1975 to discuss the optimal method or methods. Cook reported to that meeting that overall frozen cells had been disappointing and had not yielded the usually accepted 60% which one would see in a population of volunteers. Of course the D antigen may vary in strength depending on the donors rhesus phenotype and there is some evidence that women have a higher rate of response when the cells come via the placenta of a rhesus incompatible foetus. There is also a delay from the time the cells are thawed and recovered. They are transferred aseptically into sterile McCartney screwcap bottles fitted with rubber liners and injection site caps, the tops kept sterile with viscap covering for transport by B.T.S. van to the Railway Station and the onward railway journey to

Inverness. Batches of cells are thawed on any one occasion and it is often possible, depending on the red cell antigen profiles, for each donor to receive an individual, separately labelled, clearly identifiable, McCartney container. The residue of cells remaining at the Transfusion Centre are used for dialysis patients if a request has been received for that day. Full sterility checks are, of course, carried out on all of the cells injected.

To date the transport arrangements have worked very well and Dr. Cook can bring all of the recipients to Inverness Centre at a pre-arranged time.

To my knowledge this work done by Cook in Inverness is the first of its kind ever undertaken in humans and I was fortunate in being able to collaborate with him in this important venture which will be jointly published.

Haber et al (1968) reported the careful selection of fully profiled frozen cells (22 blood group factors) to minimize the chance of future immunisation in a group of thalassaemic patients some of whom entered the study with preformed red cell antibodies. They reported that no newly induced red blood cell antibodies appeared in any patient receiving thawed frozen processed red cells as well as the marked reduction in pyrexial reactions due to pre-existing leucocyte antibodies which had failed to be controlled by using packed cells or buffy coat poor red cells.

ELECTROPHORETIC MOBILITY OF RECOVERED CELLS

Electrophoretic mobility of cells is extremely sensitive, precise and directly related to the state of the cell surface and is a constant feature in any individual human. It varies from one mammalian species to another and is maintained in a saline suspension for at least 36 hours. (Angers and Rottino 1960, Zerial and Wilkins 1974).

A reduced mobility is associated with loss of surface characteristics including sialic acid, surface proteins, amino acids and sulphydryl groups (Cook et al 1961, Furubjelm et al 1969). Red cells are usually examined whilst suspended in saline or other electrolyte solutions. I thought it might be of value to examine cells before and after freezing by this technique as well as using a qualitative test of the red cell membrane sialic acid content (Greenwalt and Steane 1973). Donations of blood were collected in the normal manner into CPD plastic packs for the red cell freezing programme from healthy volunteers. Red cell saline suspensions of thrice washed aliquots were examined before and after freezing and thawing using a capillary cell Electrophoretic apparatus (Rank Bros. Cambridge).

The results are shown in the Table which gives the comparative figures for other recorded series. Taken with the morphological light and electron microscopy observations as well as the biochemical and antigenicity findings in the present work there would seem to be no loss of mobility and no loss of membrane characteristics so far as can be ascertained.

As well as determining the mobility of post thawed cells the results were compared with the same cells before freezing as shown in the Table. If anything, there is an increase in the mobility of the recovered cells but direct observation

suggests that this may be due to a coating of a non-surfactant film in the flow cell. Experiments are being done to find an easy way of removing this to achieve reproducibility. The exactly opposite effect of a slowing down of mobility was found by Angers and and Rottino (1960) when serum suspended cells were compared to washed cells. They postulated a saline elutable coating from the serum, which must be adsorbed on the red cell surface. In reproducibility studies with 60 consecutive readings, washed cells from four healthy donors showed only a variation of 0.0 to 0.10 microns. Nevertheless they used 40% nitric acid solution between experiments and weekly sulphuric acid chromate to keep the observation chamber meticulously clean. Our system may require a similar or powerful detergent washing but these observations do not invalidate the results obtained since the low resistance coating had a similar effect on the non-frozen washed cells as well as the frozen resuspended cells. Seaman and Heard (1961) recorded an increased mobility of 1.41 $\pm$ 0.04 $\mu sec^{-1} v^{-1}$, cm^{-1} of cells suspended in autologous serum but the relevance of this to the present study is not clear.

TABLE 4/40

ELECTROPHORETIC MOBILITY OF SALINE WASHED RED CELLS

Microns/sec/volt/cm at pH 7.0

Authors	
Abramson (1929)	1.31 ± 0.02
Abramson (1929a)	1.30
Furchgett and Ponder (1941)	1.03
McQuillan (1950)	1.32 ± 0.05
Brody (1951)	1.07 ± 0.02
Hartman et al (1952)	1.27
Loveday and James (1957)	1.31 ± 0.02
Bangham et al (1958)	1.07 ± 0.02
Angers and Rottino (1960)	1.32 ± 0.05
Angers and Rottino (1960)	1.27 ± 0.05
Seaman and Heard (1961), Cook (1961)	1.08 ± 0.03
Documenta Giegy 6th Edt. (1962)	1.31
Ruhenstroth-Bauer (1965)	1.27
Furuhjelm et al (1969)	1.09 ± 0.005
Seaman et al (1969)	1.08
Vassar et al (1973)	1.08
Zerial and Wilkins (1974)	1.10

Present Work (1975)	Pre	Post	Sialic acid content
1. 125670	1.08	1.07	Normal
2. 125675	0.97	1.05	Normal
3. 125725	1.15	1.21	Normal
4. 125699	1.29	1.23	Normal

STUDIES ON ALUMINIUM CONTENT OF FROZEN BLOOD

Reference has been made to Shaw's letter (1972) and to the cautious approach to using aluminium containers for frozen blood (p. 121). Parsons et al (1971) noted the association of raised concentrations of aluminium in bone biopsies from patients suffering from long standing anaemia. Values of up to 10 times normal were found, indicating a considerable accumulation probably due to phosphate depletion associated with excessive intake of oral aluminium hydroxide. Aluminium is the second commonest element in the human body (Butt et al, 1960). It is more plentiful than any other metal on the earth where it ranks third element in abundance (O_2, 50%; Si, 25.8%; Al, 7.3%; Fe, 4.18%) and comprises about 8% of the earth's crust. Despite being twice as plentiful as iron or calcium and three times that of sodium or magnesium, it has no proven physiological function nor is there any clue to its possible part in pathological states, (Seven and Johnson, 1960, Ellsworth Smith, 1928). Berlyne et al (1972) have shown in rats that aluminium salts can be harmful when given to those with pre-existing kidney damage. Aluminium compounds are widely distributed in food (Ellsworth Smith, 1928, B.P. Codex, 1973, Martindale, 1972, Food Chemicals Codex, 1972). It is generally accepted that these are not dangerous to health. It is important however to recall that any aluminium in frozen blood will be given intravenously and storage in bone might occur if any substantial quantity were to be administered (Parsons et al, 1971). Jenkins & Blagdon (1971) reported that the residual sterilizing water in their cannisters could have levels of 0.5 microgram/ml. In this respect it is quite

fallacious for Shaw's calculation that scaling up this result would mean 250 micrograms/500 ml (the volume of a unit of recovered cells). In a search of the literature I have only been able to find some reference to acute experiments in rabbits and dogs given intravenously various soluble salts of aluminium (Ellsworth-Smith, 1928). Large doses were given and proved toxic. These experiments however are so grossly unphysiological as to be of little direct value - 5cc of 0.8% aluminium sulphate every day for 30 to 50 days (2.4 mgm/l day). Others received 1.1 mgm/l day of aluminium chloride and the 'control' animals were given 1 ml sulphuric acid! Since my thesis is presented to The University of Glasgow it is appropriate to record that this same author cites two famous Glasgow Alumnii, Dr. John Glaister, Regius Professor of Forensic Medicine and Dr. Andrew Allison who concluded (1913) that 'ordinary use of aluminium cooking vessels for culinary purposes is not attended with any risk to health, or the consumers of food cooked therein'. What is of equal significance however is the reference to work of Friend and Vallance in 1922 on the influence of protective colloids on the corrosion of metals and on the velocity of chemical and physical change. They are cited as discovering that the presence of protective colloids is probably an explanation of the marked resistance to corrosion of aluminium vessels. Using the electrolyte action of 3% NaCl solution and regarding its mean relative corrosive action as 100, the addition of 0.1% gelatin reduced the action to 44.6%. This observation may therefore be the reason why the frozen red cells suspended in residual isotonic plasma with additional cryoprotectant (glycerol)

does not result in massive destruction of the cannisters or electrolytic dissociation of aluminium. The Table shows the analysis of our cannisters carried out at Colville's Ravenscraig British Steel Plant, Motherwell. The extremely high purity of the aluminium is noted. The accompanying (4/42) Table shows the levels of extractable aluminium detected at various stages of the processing of fresh red cells as well as accepted normal values from the literature. It is to be noted that all values are very low and at the limit of detection. Even the sterile cryoprotective and wash solutions contain some aluminium. Red cells themselves contain aluminium but since the method used for the analysis causes total destruction of the red cells with release of contents the figures given represent the total amount of detectable element. Post mortem studies on recipients of frozen blood, dying of their disease, as well as in life excretion studies would be of value (urine analysis) but it is likely that the method used would not prove sensitive enough for this in any reliable 'balance' study. In all cases except one (Unit No. 52094) the post recovery aluminium content of the transfused cell suspension was in fact close to the normal liquid whole blood and plasma levels. If this is true then frozen cells would represent no more of a hazard than conventionally transfused whole human blood, of which countless millions of recipients testify to the innocuous nature of such small doses of intravenous aluminium. Wrong and Swales (1970) likewise concluded that since aluminium is so abundant on the earth the enormous amounts consumed by man and animals should have revealed itself through countless

generations. Almost 50 years have passed since aluminium hydroxide was introduced into medicine (B.P. 1973), Food & Chemicals Codex,(1972), Martindale, 1972) and 30 years since its use in the treatment of peptic ulceration. Despite this no marked effects have ever been reported although very recently (Lancet, London, 1975) suggestions have been made of the possible role of aluminium in a peculiar dementia in chronic renal failure patients given alluminium hydroxide to prevent phosphate retention. This 'Dialysis Dementia' has led to a challenge to the non-toxicity of aluminium and should lead to interesting future discussion. As the Leader points out, dialysis is an artificial way of prolonging life and for this reason might be expected to have unwanted sequelae. Certainly in our experience with the thousands of units transfused, no such medical problem has been encountered.

TABLE 4/41

CHEMICAL ANALYSIS OF ALUMINIUM CANISTER

Aluminium	99.1%
Iron	0.06
Silica	0.01
Manganese	0.02
Copper	Absent
Tin	Absent
Lead	Absent

TABLE 3/42

EXTRACTABLE ALUMINIUM micrograms/gm. wet weight

(METHOD - See Appendix)

Unit No.	Prefreeze	Post Thaw	Final Product
076884	-	0.084 ± 054	0.18 ± 0.058
52088	0.074 ± 0.021	0.25 ± 0.023	0.17 ± 0.026
52094	0.19 ± 0.061	0.60 ± 0.067	0.86 ± 0.081
52174	0.077 ± 0.02	0.23 ± 0.024	0.39 ± 0.05
22633/72	-	0.49 ± 0.065	0.33 ± 0.12
024474	≤ 0.05	≤ 0.05	≤ 0.06
024526	≤ 0.05	0.07 ± 0.06	≤ 0.05
024527	0.43 ± 0.07	≤ 0.05	0.10 ± 0.07

Sorbitol/glycerol	0.032 ± 0.028	
Normal Saline	0.036 ± 0.029	
Sorbitol Wash 1	0.38 ± 0.033	
Normal Blood	0.15 (0-04)	*
	0.13	**
	0.32	***
Normal Red Cells	0.07 (0-0.17)	*
	0.16	***
Normal Plasma	0.46 (0-0.88)	*
	0.44	**
	0.24	***

* Values from Spector, W.S., Handbook of Biological Data. W.B. Saunders Co. London.

** " Bowen, HJM (966) Trace Elements in Biochemistry p.81

*** " Documenta Giegy, Scientific Tables: p.570 6th Ed.

See also Snyder et al (1974)

A.T.P. STUDIES

A number of authors have shown that ATP is essential for red cell viability (Mitchell 1976 for review). Others have assayed this important substance in cells before and after freezing as shown in the Table 4/43 and at varying time intervals after resuspension.

From this information in the literature it seems that ACD and CPD anticoagulated donations withstand freezing and thawing equally well with no significant loss of ATP activity. Nevertheless little was known of the metabolic integrity of the recovered cells until 1970 when Rowe et al published an exhaustive set of results designed to show just what changes if any occurred with freezing and thawing. They showed that red cells either fresh or stored up to 21 days subjected to sub-zero temperatures and processing by the low glycerol-rapid freeze method and subsequently recovered in autologous plasma with storage at $4^{o}C$ up to 21 days, had concentration of ATP comparable at all times to those of their non-frozen, non processed counterparts.

It seems too that there is no great difference with different methods of freezing and recovery and that certainly for transfusion shortly after recovery the red cells should be able to maintain membrane stability and integrity.

ATP µmoles/g Hb

Author			Pre-freeze	Post Thaw	Remarks
Prins and Loos	(1965)	ACD	1.05	1.01	mmoles/l cell
Krijnen et al.	(1964)	ACD n=4	1.13	1.01	mmoles/l cell 50% activity after 3 weeks storage in autologous plasma
Valeri et al	(1966)	ACD	-	15.9±0.2 (4 days)	µmoles/g Hb
Rowe et al	(1968)		"unchanged"	3.15	
	(1970)		3.35		
Derrick et al	(1969)		2.8±0.55	1.51±0.42	µmoles/g Hb
			2.56±0.43	1.50±0.34	µmoles/g Hb
Krijnen	(1970)		"unchanged"		
Prins and Loos	(1970)	ACD	1.11±0.19	1.75±0.17	mmoles/l cell
Prins and Loos	(1970)	CPD	1.23±0.08	-	mmoles/l cell
Tullis et al	(1971)	ACD	3.00	2.52	µmoles/g Hb
Button et al	(1972)		2.90	-	µmoles/g Hb
Derrick et al	(1972)	ACD	3.13	3.61	µmoles/g Hb
Gibson	(1972)	ACD	2.7	3.2	µmoles/g Hb
		CPD	2.8	2.8	µmoles/g Hb
Akerblom	(1974)	ACDA	3.46±0.57	3.48±0.56	
	(1974b	ACDA	3.5±0.9	3.8±0.9	
Elutramatic Wash					
Akerblom	(1974b		3.46±0.47	3.46±0.54	cytoagglomeration recovery
				3.80±0.63	batch wash recovery

Table 4/43. Adenosine Triphosphate Content of frozen blood.

NUCLEOSIDE UTILIZATION

In considering nucleoside utilization it has been similarly established (Rowe et al 1970) that recovered red cells can utilize nucleosides just as well as non-frozen cells as an alternative source of energy substrate. Krijnen et al (1964) used adenosine and inosine and it has been suggested that older blood (up to 21 days) could be "rejuvenated" in preparation for freeze preservation. (Valeri and Zaroulis 1972; Moore et al 1973; Valeri 1975; Mitchell 1976 for review). Gibson et al (1972) have demonstrated that frozen cells could be stored after deglycerolization for up to 2 weeks when resuspended in a solution which is supplemented with adenine 0.5 μmol/ml blood.

Ginzburg, Sinkhova and Talskaya (1971) resuspended washed cells recovered from liquid nitrogen for varying lengths of time up to 3 years and showed that these frozen recovered cells have their storage time (post recovery) increased for up to 5 days by the addition of inosine and adenine to the final resuspension fluid. Unfortunately, this Russian worker's paper does not give details of which cryoprotectant solutions were used but since their address is given as Central Institute for Haematology and Blood Transfusion Noroxikovski prospect 4, Moscow, one presumes that they used the 16-20% polyvinyl pyrollidone method of Vinogradov-Finkel and his colleagues (see p142

Gilcher et al (1968, 1969) carried out a study on the post thaw stability of CPD frozen red cells and found that the recovered cells showed active glycolysis, gradually decreasing ATP and glucose levels with fall in pH and rise in inorganic phosphate to a degree similar to that observed in conventional CPD banked red cells. Chromium tagged survival studies in vivo with recovered cells resuspended in autologous plasma with or without adenine during the pre-transfusion storage at 4°C showed an average of 90% survival on the day of processing, 70% at about 15 days and prolonged useful survival to about 21 days in recovered cells fortified with adenine.

FROZEN BLOOD

VOLUME II

Thesis presented for the degree of Doctor of Medicine

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by

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"What miracle of weird transforming
Is this wild work of frost and light
This glimpse of glory infinite".

Wittier, The Pageant.

SURVIVAL OF FROZEN CELLS

Mollison and Sloviter (1951) first observed that human red cells mixed with glycerol could be frozen to -79°C for 2 or 3 hours in relatively large quantities, thawed deglycerolised with little lysis and then transfused with normal in vivo survival. Storage experiments showed that after 6 months at -79°C the survival of recovered red cells was almost unimpaired. Other investigators used higher concentrations of glycerol and Brown and Hardin obtained 95% recovery of cells frozen in 5.2M glycerol at -79°C. Survival of 6 months stored cells was only 60% at 24 hours and thereafter it was normal. Clearly considerable loss in the first 24 hours post transfusion had occurred but this was not entirely due to failure of deglycerolisation since Jones et al (1957) showed that prior treatment with too much glycerol had a damaging effect on cells subsequently frozen. Some post transfusion loss of cells occurred with recovered cells stored for more than 21 months at -79°C in 3.0M glycerol (Chaplin et al 1957). Thereafter survival was normal irrespective of the time spent in store at -79°C. At -79°C the electrolyte content of the suspending medium is of little importance although with storage at -20°C this is of importance. (Chaplin and Mollison 1953). pH adjustment to about 7.0 greatly improves the maintenance of viability (Chaplin et al 1954). Post transfusion survival of human red cells stored in glycerol - citrate - phosphate - medium (final glycerol 3.2M) at -20°C diminished as the period of storage increased. After 6 months of storage, the survival of the cells at 24 hours was 75%, after 2 years it was about 45% (Jones et al 1957). For storage at -45°C the final glycerol had to be 4.5M to minimize haemolysis.

24 hour post transfusion survival of red cells stored at -45°C for three months, deglycerolised and stored at 45°C for 10 days before transfusion was approximately 70%.

Extensive clinical evaluation of red cells processed by the ADL centrifuge bowl system have been reported (Tullis et al 1958; Haynes et al 1960; Haynes et al 1962; Goodale 1963; O'Brien and Watkins 1960; O'Brien et al 1961). The average 24 hour survival was 85% with normal T/2. Between July 1957 and July 1962, Haynes et al (ibid 1960, 1962) transfused 2334 units of deglycerolised blood suspended in 5% albumin to 1327 patients. Fewer febrile reactions, cardiovascular pressor effects and urticaria were noted than occurred with ACD preserved blood. In addition there was a suggestion that the incidence of icterus was reduced. O'Brien and Watkins showed normal gas dynamics (O_2 uptake and oxyhaemoglobin dissociation) both in vitro and in vivo. They also showed that the frozen recovered cells could be used for vascular surgery and extracorporeal circulation (1961). Thawed deglycerolised red cells resuspended in thawed heparinized plasma showed no significant changes in gaseous properties when compared to recirculated fresh heparinized blood after prolonged circulation through a pump oxygenator.

In 4 patients who had significant blood volume replacement (30-500% of total blood volume) there were no untoward side effects. In particular there were no increases in haemorrhagic phenomena despite the use of 5% albumin for resuspension. Although their patients on frozen cells did as well as these on banked ACD blood, O'Brien et al (1961) did report the appearance of a peculiar syndrome after

cardiac by-pass associated with rising blood urea and failing renal concentrating function together with a large urinary output. This seemed related to length of perfusion and was possibly but not conclusively due to haemoglobinuria. In 1962 came the report of haemoglobinuria with or without acute renal failure due to resuspending cells in outdated 5% albumin (Valeri and Henderson 1964; Tullis et al 1963). These later authors showed that any 25% albumin solution would become unstable when diluted to 5% in the presence of glucose and that the instability was greater when the source of albumin was derived from outdated supplies. Studies on in vivo survival in these various conditions showed that autologous plasma was superior to 5% indated albumin medium which in turn was superior to 5% outdated albumin medium (Valeri 1965).

Huggins (1965) reported, by his cytoglomerator process, 87-95% survival of red cells labelled with radioactive chromium 24 hours after transfusion. An in vitro processing loss of 10% was reported (3% haemolysed, 7% intact cells). Supernatant haemoglobin was 0.29% of the total haemoglobin transfused. He reported transfusing 1081 units to 124 patients (average 8.7 units per patient) without difficulty.

Valeri (1966), Valeri and Bond (1966) at the Chelsea Naval Hospital Boston, Massachusetts reported on a clinical evaluation of glycerolised frozen cells recovered by agglomeration. Complement coated cells survived normally in healthy volunteers. No deleterious effects were demonstrated by in vivo or in vitro studies on blood stored for one month to two years although the mean loss

was 22% in vitro. Post transfusion survival was 85-96%. Post thaw storage at 4°C in autologous plasma was up to 5 days but no advantage was claimed for this since the bacteriological hazard of prolonged storage beyond 24 hours out-weighed any benefit in their view. They recommended storage in saline-glucose and transfusion within 24 hours. Since their methods require mechanical refrigeration they studied the survival of cells deliberately kept at higher temperatures +4°C, -20°C and -30°C in order to stimulate a power failure. Excessive in vitro losses of cellular haemoglobin and unacceptable post transfusion survival of chromium labelled cells were observed for autologous red cells stored at +4°C for longer than 24 hours, at -20°C for longer than 3 days and at -30°C for longer than seven days. These findings stressed the need for maintenance of temperature at -80°C and showed that a supply of solid carbon dioxide should always be available on hand in case of electrical power failure. In my view this seems an unacceptable risk and formed part of the reasons that we decided to develop a frozen cell bank independent of the power supply (see Vol, I). de Verdier et al (1963) and Valeri and McCallum (1965) suggested that their in vitro studies showed that freezing and thawing preferentially preserved the "young" cells in any unit of donor blood and that in this way, the processing losses could be offset by provision of a younger population of surviving cells.

Using the Ashby differential agglutination technique in one patient with erythroid hypoplasia Perrault et al (1967) showed that cells recovered from a high glycerol -80°C bank survived normally. Later using radio-chromium in

9 subjects they showed normal survival of frozen autologous cells with T/2 averaging 35.5 days (29-40).

Recently De Furia et al (1974) have suggested that thalassaemia patients receiving maintenance frozen blood transfusions may still show some metabolic abnormalities because of the high oxygen affinity cells (containing HbF) produced endogenously.

CLINICAL EXPERIENCES WITH FROZEN BLOOD

In January 1966, the U.S. Navy established a Huggins type frozen cell bank at Danang aboard the Hospital Ship, U.S.S. Repose Vietnam to supplement the conventional ACD preserved blood (Moss, Valeri and Brodine 1968; Valeri, Brodine and Moss 1968). Haynes et al (1960) had previously shown the use of large volume infusions in major surgery in civilian practice. Observations showed the method to be feasible and operational in a combat zone (43 patients received 307 units of frozen blood.) Later Valeri(1969) reported 3000 units had been given to severely wounded service men without ill effects, even on patients who received massive amounts of frozen blood. (one patient received 41 units). A decline in recipient platelet counts was considered no more than would have occurred with liquid banked blood and was attributed to depletional and dilutional effects.

Haemoglobinaemia was observed following multiple transfusion of frozen cells. This was considered due to (a) the residual amount of supernatant haemoglobin in each unit and (b) the lack of any protein binding since the final suspension fluid contained no protein. No renal failure occurred however and renal function remained good in part probably due to the rapid forward treatment of casualties with large volumes of electrolytes (Ringer - Lactate and mannitol/sodium bicarbonate).

Following this successful demonstation of the clinical acceptability and technical feasability the U.S. Navy established a further frozen cell bank at Clark Air Force Base, Philippine Islands to provide selected red cells for uraemic patients undergoing haemodialysis.

Valeri et al (1970) reported the use of frozen cells in priming a heart lung machine during cardiac surgery and reported no differences from conventional blood at 5 days and 4°C storage. No significant differences could be seen in coagulation, renal function and haematologic measurements. Earlier success was reported by Haynes et al (1960) and O'Brien and Watkins (1961). Huggins (1970) reported 141 by-pass operations using frozen blood and Rudear reported 12 such procedures (p36.).

Frozen autologous red cells (two units) stored frozen and later used for elective obstetrical and gynaecological operations were reported by Daane, Valeri and Brodine (1969). Perrault et al (1967) reported that 21 patients were given frozen cells without adverse clinical reaction; all showed rises in red cell haematocrit commensurate with the amounts infused (1-5 units). One patient showed transient haemoglobinuria but this patient's haptoglobin levels were zero before and after transfusion.

In an Editorial note to the paper by Perrault et al (1967) describing the possible use of frozen cells for auto transfusion it is stated that according to the National Headquarters of the Jehovah's Witnesses in Canada, blood taken from an individual, frozen, stored and later given to the same individual would violate their beliefs. The author has had occasion to arrange for auto transfusion in a Jehovah's Witness in West Scotland and certainly did not encounter such a problem. It would also seem to conflict with the reports of major cardiac surgery and auto transfusion in other reports. Gerst et al (1967), Akerblom and Hogman (1968) have recorded cases of auto

transfusion. In Akerblom's case regular small donations of blood from a renal dialysis patient were stored frozen and later used as autologous blood during gastrectomy (Hogman and Akerblom 1970).

Prins et al (1966) and Krijnen et al (1966) and Haynes et al (1960) reported normal survival in a few healthy adults. Krijnen et al (1968) reported auto transfusion and homologous transfusion in 3 normal subjects and three patients, and Akerblom et al reported normal 24 hour survival in 3 cases (> 93%).

Huggins (1965) reported 673 units of frozen cells had been given to 94 patients. Haynes et al (1960) reviewed the use of 1014 units resuspended in 5% albumin or autologous plasma after varying periods of storage up to 44 months. Two hundred and ninety patients received 1-3 units, 65 received more than 4 units and 5 received up to 16 units during a single surgical procedure. None showed any coagulation failure despite the lack of clotting factors in the 5% albumin product. Coagulation factors in the patients remained within normal limits for fibrinogen, prothrombin, platelets, bleeding time. They noticed the ten fold reduction of allergic (0.5%) transfusion reaction and the absence of any homologous jaundice - although the disease was endemic among their donor population. Resuspension of cells in autologous plasma resulted in one case of homologous serum jaundice.

Tullis et al (1958) reported no adverse effect in 33 recipients and recorded no evidence, haemoglobinuria or bilirubinaemia. Supernatant haemoglobin were as predicted and recovered red cells showed normal in vitro oxygen carrying capacity.

Strumia and Strumia (1966) reported survival results in 12 cases using Dextran/albumin stored cells. Thompson et al (1966) reported the use of PVP protected frozen cells in 21 monkeys.

Sproul et al (1965) reported that to date 3500 units of frozen cells had been transfused to 1120 patients and Valeri (1969) estimated that up to that time about 10,000 units of blood had been recovered by Huggins method and successfully transfused in civil and military practice. Huggins and Grove-Rasmussen (1971) gave details of 2273 patients transfused and commented that no elective operation at their hospital had to be cancelled for lack of blood:-

Open heart surgery	874
Kidney Tx dialysis	429
RBC antibodies	223
WBC antibodies	88
Substitute for ACD packed cells	659
Total Jan 1- 30th June 1969	2273

Gradually increasing usage had occurred since 1965 with diminishing outdating figures:-

	B/F units transfused	Frozen Units transfused	Outdating %
1965	27958	736	9
1966	32134	1155	8
1967	36483	1538	7
1968	44562	2295	5
1969 (Jan-June)	24534	2273	< 4.0

Recovered cells showed the following (average for 50 units): Na^+ 84.3 meq/l, Cl 85.2 meq/l, K^+ 1.9 meq/l, citrate 0, total Protein 0, WBC 650/cmm, anti-A 0-1/4, anti-B 0-1/4.

They considered the absence of anti-A and anti-B as well as protein to be appealing as a return to universal donor and decided to use group O rhesus negative, O R_1R_1 and O R_2R_2 to extend the concept to the rhesus system. Further testing was done for Kell and Fy (a+b+) (see rare donor definition Rowe 1970 p 33.). Removal of leucocytes they considered beneficial and permitted the uneventful transfusion of leucocyte sensitised patients. For the previous 6 years (1963-1969) all dialysis patients and renal transplant patients at Massachussetts General Hospital had had leucocyte poor frozen cells and no case of serum hepatitis had occurred which could be attributed to the use of frozen cells (Huggins 1965). Work by Summida from National Medical Centre at Fukuoka, Japan was cited where 825 units of frozen blood had been transfused showing a normal background attack rate of 15-16 % in patients not receiving the frozen blood but only conventional ACD units stored at 4°C. Huggins (1965) was anxious to introduce frozen cell banking for two reasons.

(a) indefinite storage, ease of shipment and alleviation of the outdating with added ability to store rare groups and autologous donations.

(b) removal of unwanted components such as potassium, anticoagulants, fixed acid was useful in the case of patients with uraemia, congestive heart failure and the metabolic derangements that take place during multi unit transfusions. Removal of donor plasma eliminated urticarial reactions, allowed group O blood to be used for all patients and might reduce the incidence of serum hepatitis. These last two attributes prompted him to introduce frozen blood for all renal allo transplant recipients and potential recipients (see p 80).

Vinograd-Finkel and Kiselev (1971) in reviewing Russian work stated they had successfully transfused low glycerolised (15%) blood stored from 1 to 5 years in liquid nitrogen into 1043 patients including dialysis and cardiac by-pass cases with no untoward effects. Some received 1000-2000 ml and supernatant haemoglobin in the suspension was 13-100 mgm/dl. Similarly (1971b) using PVP they reported favourable results in humans and animals with recoveries of 96% and supernatant haemoglobins up to 195 mgm/100 ml. They considered that transfusion without washing would allow 500 ml of processed blood to be given before the haptoglobin mechanism became saturated with the free haemoglobin. In vivo survival was about 80% at 24 hours post transfusion. They considered that low molecular weight PVP would not bind to plasma proteins and would be rapidly excreted. Federova, Ivanova and Semenova (1971) wrote of transfusing 270 units of blood frozen and stored over 5 years by Vinograd-Finkel's method for patients having physical reactions to conventional transfusion. Miura et al (1971) using the Huggins cytagglomerator for recovery of glycerol preserved cells found the immediate pre-infusion haemoglobin in the saline suspension of recovered red cells to range from 80 to 220 mgm/100 ml (average 115 mgm/100 ml) (contradicting his figures quoted in vol.1.) with recovery of 84-90% of cells whose in vivo survival (^{51}Cr) was 80-93% at 24 hour post infusion with normal T/2 life. They commented on the value of low potassium levels in the infusion product for dialysis and cardiac patients. As an added bonus they quoted that only 0.5% of Japanese are rhesus negative and that this makes acquisition of rhesus negative blood quite a problem.

Frozen cell banking had allowed stockpiling of this "rare" Japanese blood group.

In an excellent paper on low glycerol rapid freezing method Rowe et al (1971) describe their clinical approach to patient care following successful in vitro studies. They give the usual reasons for starting a frozen cell bank and describe the use of 1100 frozen units from a bank stored donations divided into rare blood, fully typed blood, partially typed blood, blood held for a specific hospital, blood held for a specific patient and blood for autologous use. In all 263 patients received frozen cells with no difficulty up to the end of the review period June 1969 (Table 5/1). Blood up to 2½ years old had been used and 269 patients in other parts of U.S.A. had received cells from this bank:-

Transfusion Category	No. of Patients	No of units transfused
Auto-transfusion	9	16
Exchange transfusion	8	9
Leucocyte and plasma protein antibodies	23	99
Surgery (organ transplant, open heart surgery, hepatitis risk etc.)	98	151
Anaemic (Thalassaemia major)	18	27
Cooley anaemia	22	560
Red cell antibodies		
rare blood	25	67
multiple antibodies	60	243
	263	1172

Table 5/1

In discussing these transfusion requirement categories they instance 50 units in reserve for auto-transfusion (a) in patients whose needs included rhesus null, -D-, Vel, Tj^a, and Knops. (b) in those where it would be impossible

to find a donor where the patient has been immunised to many high-frequency antigens, or there were religious objections by the patient to homologous transfusion and (c) as a means of minimising the risks of post transfusion hepatitis.

They had used frozen blood for exchange transfusion where fresh bank blood was not available because a multiplicity of antibodies was involved or a rare antibody against a common antigen was responsible for the haemolytic state in the newborn eg. U negative blood as reported by Puno et al (1967).

They considered that pyrogenic reactions to bank blood due to leucocyte of plasma protein immunisation could be prevented by thawed recovered cells as had been reported by Haber et al (1969), Jones et al (1968). These findings have since been confirmed by a large number of authors and it is established that the best and most efficient way of freezing stored blood of leucocytes and plasma components is by differential freezing and thawing with subsequent washing during the recovery phase (Review, Mitchell 1976). Because of the importance of such immunisation in patients awaiting renal transplant they further stated that such patients should receive frozen blood free of immunogen. For a similar reason they considered the risk of transmission of hepatitis virus to be worth trying to avoid by using multiply washed recovered frozen cells since Prince (1968) had shown that the antigen (hepatitis) (HBsAg) was located in the plasma. Tullis and Lionetti (1966) noted that among 2000 recipients of frozen blood there were no cases of icteric hepatitis whereas two cases occurred in the first

300 recipients of similarly frozen and processed cells resuspended in their original autologous plasma although they recognised that probably as many of a hundred times more cases of non-icteric hepatitis might occur, (Hampers et al 1964; Cooper et al 1966) only recognisable by deviations of liver function tests from normal. In a later, more detailed "double blind" study Tullis (1970) (over 4½ years) found 4 cases of histologically proven hepatitis (one icteric, three anicteric in a control group of 104 recipients receiving 422 transfusions (median 3) of frozen stored and recovered red cells resuspended in autologous plasma - an incidence of 9 per 1000. No hepatitis occurred in a group receiving 623 transfusions (median 4) of similarly frozen and recovered red cell resuspended in albumin. Patients were followed up for 6 months and previous cases of jaundice, hepatitis, liver disease, prolonged undiagnosed fever and infectious mononucleosis were excluded from the trial as well as any showing recent or current biochemical evidence of disordered liver function (SGOT, SGPT), history of receiving any human product or tattooing within the previous 6 months. Neither the patients nor the physicians doing the follow-up or evaluating the histological liver biopsy specimens knew which resuspension medium had been used. He considered that the attack rate in the non-plasma group of frozen cells was zero and anticipated that the same benefit could be anticipated with resuspension in hepatitis free saline. He presumed that hepatitis virus was physically displaced in the washing and that glycerolization, freezing long term, cold storage and thawing have no effect on virus activity. Of course he had no evidence of this and recognised that similar

studies were needed for all existing methods of preparing frozen cells in bulk for transfusion purposes. He went further and cautioned that his methods' success did not imply that any other extracellular (saline) wash of fresh ACD red blood cells would render transfusion units hepatitis free (Hinman and Tullis 1971). As far as he was concerned the findings applied only to frozen cells and that other investigators testing other washing or freezing systems could not automatically expect similar benefits (Medical News 1970). Janeway (1970) considered that this type of study would need to be made in the case of all other techniques before they could be generally accepted unless it could be shown conclusively that donors carrying the virus of post transfusion hepatitis can be successfully eliminated by testing for the infective agents of hepatitis. No such surveys of other techniques have been conducted and are unlikely to be done since the advent of almost Universal sensitive screening of donations for HBsAg which were not available to Tullis during the period of his study for 53 months from September 1965 until February 1970. Werch et al (1971) studied seven known HBsAg positive donor samples by counter immunoelectrophoresis and microtitre complement fixation tests. Freezing and thawing with agglomeration recovery showed hepatitis antigen in five of the seven discarded wash solutions and in only one of the seven supernatant reconstituting saline solutions used in the transfused product. They considered that the process was purely simple dilution. This donor had the highest level of antigen on the initial screening (1/10250 by C.F.). As early as 1966 Huggins had reported to the

American Society of University Surgeons annual meeting at Harvard Medical School that 2229 units of frozen cells had been transfused in his hospital (Massachusetts General) without any case of hepatitis being evident. He expressed the view that "if this trend is borne out by additional experience to the point of statistical significance it would make washing of all blood mandatory before its administration". By 1970 (Huggins 1970) he was still reporting that serum hepatitis kills 5000 patients per year in U.S.A.

Interesting work reported by Miura et al (1969) at the 12th Congress of the International Society of Blood Transfusion in Moscow showed that blood deliberately infected with four different viruses could be considerably reduced in the processing of freezing, thawing and recovering and that such a reduction would probably account for the marked reduction in post transfusion hepatitis. That the result might not be entirely due to physical dilution and removal by washing virus away in the supernatant to subinfective levels is suggested by an interesting experiment described by Damjanovic at the 1972 meeting of the Low Temperature Society held at the Biological Research Centre, Ministry of Defence, Porton.

In this it was shown that Simmian virus which was normally haemolytic could be rendered non-haemolytic by freezing in liquid nitrogen and later recovery. Against this however are accounts of maintaining virus infected cell lines stored in liquid nitrogen for subsequent recovery in order to compare with newer infected strains allowed to grow on from the original and to adapt and alter in the process. These later cultures can then be compared to the original so called "stabilate" culture.

Huggins (1970) commented on the epidemics of serum hepatitis which occur in patients and personnel in dialysis units throughout the World. Huggins (1974) in reviewing our 50,000 units of frozen cells recovered stated that despite a Federal Drugs Administration enquiry no cases of post transfusion hepatitis had been found. Kingsley (1973 Personal Communication) quoted Kliman that 15,000 units of frozen cells had been prepared by bowl washing centrifuge (Haemonetics) with one case of post transfusion hepatitis in a careful follow-up of recipients. Haemonetics system was very efficient compared to cytoagglomeration. Carr et al (1973) reported that the exclusive use of frozen cells in their dialysis unit markedly decreased the incidence of hepatitis.

The Rosenheim committee in their report (1973) on hepatitis in dialysis units commented favourably on the evidence sufficiently to encourage those few workers in United Kingdom, who possessed frozen blood banks to advocate the use of frozen cells in dialysis patients as a step in the right direction. Meetings were held in Glasgow Royal Infirmary with Professor A. Kennedy, Dr. D. Briggs and Dr. A. MacDougall to discuss those matters. Minutes of these meetings are included in the appendix. The outcome, briefly, was that all dialysis patients would receive frozen blood for planned transfusion and that a recommendation would be sent to all referring physicians in the West of Scotland that any patient who might require dialysis should be considered for frozen cell transfusion if transfusion was required. Such requests would be channelled through the Regional Blood Transfusion Centre

where the frozen cell bank is located.

Other workers have deliberately frozen blood known to be infected with hepatitis virus but none to my knowledge has had to transfuse it after recovery. Some workers have studied the problem in vitro but little is known of these experiments since they have not been published (Blagdon, Winnick 1975, Personal Communication).

Using the low glycerol rapid freeze technique, Bethel (Personal Communication 1975) froze known radioimmune-assay positive HBsAg donors' blood. Recovery by thawing and serial centrifugal batch washing showed that the Antigen (HBsAg) was only detectable in the first or second discarded supernates and not in the final product.

Umlas (1975) cited a Personal Communication from Valeri and Contreras that washing decreased the antigen associated with hepatitis to below detectable levels in the unit, although the antigen may be detectable in early aliquots of the waste fluids. These same workers showed that units of blood which are negative for HBsAg C.E.P. but positive for RIA when washed, became negative by RIA even when the waste fluids at the end of the wash was taken and concentrated 100 times. The potential advantage of washing becomes more important in view of recent reports of transfusion acquired HBsAg positive hepatitis caused by HBsAg negative units of blood (Hollinger et al 1973; Roche et al 1973; Sandler et al 1973). Schlaak et al (1975) have reported similar findings of decreased HBsAg titres after repeated freezing and thawing.

Lo Grippo (1964) before present day knowledge of hepatitis viruses became established used naturally and

artificially infected red cells(with animal viruses.) He showed that some viruses could simply be washed away but others remain firmly adherent by adsorption to red cells. It will be of interest to see if human viruses behave in this way and if so what will be their significance.

Our own frozen cell bank contains a few units which after freezing were shown to be positive for virus HBsAg by a sensitive radioimmunoassay technique. These have not been used.

Huggins and Grove-Rasmussen (1973) at the Washington meeting of the International Congress of Blood Transfusion showed highly suggestive evidence that a unit of frozen cells had transmitted the infection since the platelets from the same donor were shown to have caused the disease in a recipient. This was after they had used 20,000 units of frozen blood without any reported case. The patient received two units of frozen deglycerolised cells and two months later was mildly jaundiced but recovered at home with expectant treatment. Another patient who received two units of platelets from these same donors developed mild hepatitis. They used CIEP methods of detection and felt that the combined clinical use of frozen blood and hepatitis screening can minimize the incidence of post transfusion hepatitis.

Studies on the use of frozen cells to reduce the incidence of some allergic responses to transfusion in multiply transfused patients have been reported (Akerblom and Hogman 1974). Dramatic improvements were described by Rowe et al (1970, 1971) in the management of children suffering from thalassaemia major whose prevailing

history was one of severe reactions at each infusion, due to sensitisation by past infusion of bank blood including buffy coat poor blood. Similar results have been reported by Stewart and Blagdon and Jenkins in such children attending a Thalassaemia Unit in London (Personal Communication). Indeed I recall Professor Stewart describing graphically how such children used to cringe with fear at the further end of the bed whenever the blood arrived! (Personal Communication). Nowadays these same patients and their parents demand frozen blood. Reference has already been made to the first Glasgow patient to receive blood (vol,1). On the day she received her first frozen cells she described in broad Glaswegian terms how unwell she usually felt after every transfusion!

Lullis et al 1958), Hayes et al (1960) and Haber et al (1968) reported on the use of frozen blood in multiply transfused patients experiencing severe pyrexial reactions to conventional liquid stored blood. Haynes et al (1960) recorded that frozen cells had made transfusion possible again in patients who previously had been unable to receive blood due to severe reactions. This will be further discussed under the section on removal of leucocytes (p 38).

Frozen cells have been of value in the management of patients immunised against specific high frequency antigens or against multiple, lower frequency, red cell antigens (Miles et al 1968; Rowe et al 1969). Rowe et al (1971) reported the supply of more blood to patients who require blood which is negative for the high frequency antigens Yt^a, Lu^b, k, U, Kp^b, R'/R', Vel : 1-2, Gerbich,

Chido. They constructed a table based on the known frequencies of blood group phenotypes published by Race and Sanger (1975) showing the number of units of fresh bank blood that would have to be screened to provide the requirements for such patients. (see Table). A glance at this table shows how futile it would be to try to find compatible donors for some patients in these categories with either rare single or common multiple antibodies in their sera. They described one patient with seven antibodies E,K,M,Le(a),Le(b),Fy(a).

Huggins (1970) giving a non-usage rate of 5-30% of blood due to outdating commented that blood could be thrown away one week whilst it could be in critically short supply the next. In the two years January 1967 - December 1968, 8205 units of blood were frozen and stored and 7608 units were recovered, administered to 975 patients. Haemoglobin and haematocrit values showed a persistent elevation and major surgical procedures were performed effectively and safely.

Grove-Rasmussen and Huggins (1965, 1968, 1970, 1971, 1973) based on the study of 1½ million transfusions in 18 Centres and multiple antibodies of clinical importance have calculated that three specific categories of group O red cells stored in the frozen bank and freed of anti-A and anti-B before transfusion can eliminate the possibility of haemolytic transfusion reactions due to ABO incompatibility and virtually all combinations of Rhesus, Kell and Duffy systems. These groups are:

1. a O rr K- Fy (a-b+)
   b O rr K- Fy (a+b-)
2. a O R_1R_1 K- Fy (a-b+)
   O R_1R_1 K- Fy (a+b-)
3. a O R_2R_2 K- Fy (a-b+)
   b O R_2R_2 K- Fy (a+b-)

Further subtyping of these carefully selected donors for MNS s, P, Le^a, Le^b, Jk^a, Jk^b, Lu^a, Lu^b antigens makes it possible to provide compatible blood for patients with any single antibody and virtually any combination of multiple antibodies within all nine major blood group systems. As well as this advantage on the red cell serology the selective removal of leucocytes and platelets is a great value in patients who have antibodies to these and elimination of the viable histocompatibility antigens makes frozen cells their "choice" for transfusion of all organ allo-transplant recipients and chronic dialysis patients. No case of viral hepatitis occurred among 30 Centres who had collectively transfused 30,000 units of frozen blood. (Huggins 1970). 141 patients underwent open heart surgery on the first half of 1969 according to Huggins but somewhat paradoxically platelet concentrate (unfrozen) were used to correct platelet deficiency.

Likewise Rowe et al (1970, 1971) recorded that from the beginning of their frozen cell scheme they characterised blood as the following antigens: A B, (O), D C, Ec.e, P, Kk, K^a, Le^a, Le^b, MN Ss U, Lu^a, Fy^a, Fy^b, Jk^a, Jk^b, and whenever possible high frequency antigens such as: Yt^a, Js^b, U , Ge, Lw, Co^a, Kp^b, k, Lu^b, and Gregory. Autoanalysis was used to screen suitable donations. They froze all units that had known antibodies (p111) and any without pilot tubes. In three years they had accumulated a current stock of 3500 units. These were available for a wide range of clinical situations and, like us, as they gained experience they realised that not all units need to be fully typed in order to meet transfusion requirements. Consequently they began to store

routinely typed blood. Since this introduced a selective element into the various categories of donation (p 32) , they recorded the information on a data processing code system using IBM computer punch cards. Each card gave donor information, date frozen, blood group phenotypes and freezer location. Our own system is less costly than this system but we believe equally efficient for our needs at present (see p 54). Our system also contains more useful data such as the information about preparative fluids and their quality control, hepatitis antigen testing which is not recorded on the New York blood centre card. We prefer to search the inventory each time a problem arises and to pick out the relevant donor card with all available information at hand during the decision making part of any problem.

Rowe et al (1970) gave a detailed description of their inventory of red cell types based on the extent of red cell phenotyping and clinical needs of patients and although it is somewhat repetitive it is useful to identify the following headings.

Partially typed blood. All units are typed for ABO and rhesus groups and sometimes other blood group systems. Screening is done by autoanalyser and checked manually and any donations with antibodies in the plasma are checked to ensure absence of the corresponding antigen on the red cells. He considers that such blood has a limited role for solving antibody problems and are used for routine use for patients with a single antibody, c or e negative and immunised to leucocytes, platelets or plasma proteins.

Fully typed blood: most of these are typed for ABO, rhesus, P Kell, Lewis, MN, Lutheran, Duffy and Kidd. He

considers that they are particularly useful for complex transfusion problems because they are pre-typed for 20 or more antigens and available for patients with multiple antibodies.

Blood for a specific patient or specific hospital can be laid aside either because the treating hospital has no freezing facilities or there is a delay in accumulating enough blood of a rare type for a particular patient or there is a waiting period for sufficient donations of autologous blood for freezing and subsequent recovery either on the grounds of extreme rarity or religious objection to transfusion (Jones et al 1968). Everyone who now has a frozen cell bank has a tendency to indulge in the "magpie" cult of friendly competition as to who can accumulate the most rare donations. With the passage of time however these lists of rare donations will be circulated and as more centres develop the skill and technology for recovery work, such "rare" donations may be despatched all over the world whilst still frozen without the sending laboratory having to recover the cells and send them as $4^{\circ}C$ stored recovered cells which when they arrive although of transfusable quality, might not be required since (a) the patient may have died or his condition ameliorated, (b) the procedure may be postponed for some minor intercurrent reason (see Logistics Section for a more detailed discussion of their points). Frozen blood should not be thawed until it is confirmed that the units are compatible with the intended recipient's serum. For planned procedures, Rowe et al (1970) recorded that recovered cells matched to samples from patients referred to them had been despatched all over the United

States. Up to February 1969, 924 units of blood had been supplied to 236 patients. In eight cases (Miles et al 1968) recovered cells were used for exchange transfusion because fresh blood was not available and in a case of haemolytic disease due to anti-U, Puno et al (1967) used 'U' negative frozen bank blood. Thirteen children with thalassaemia major had received over 400 units of frozen blood, some children having had up to 70 units each. These patients, with known leuko antibodies and allergy to plasma proteins, did not suffer any pyrogenic reactions such as they had experienced previously.

Based on known gene frequencies it can be calculated the number of donors one would need to screen in order to find compatible blood for patients with multiple antibodies. Such an approach was used by Rowe et al (1970) to calculate that blood required for a patient with seven antibodies to Ms, Kell, E, Fy^a, Le^a, + Le^b would have required a search of 1000 donors to find one suitable. They had one in frozen stock and were able to obtain a fresh donation from the same donor to give the two units requested. With these predictions however there is always the danger that a failure might occur at the processing stage so even if one is committed to using frozen cells it is as well whenever possible to have additional units standing by either in frozen store or as liquid fresh blood just as one would do in any conventional banking difficulty.

Hanson et al (1972) reported the use of frozen cells in continuous recirculation for 3 hours in the bubble oxygenator of a cardiac by-pass machine. Supernatant haemoglobin and serial osmotic fragility studies showed that when the cells were resuspended in plasma they were more stable than saline suspended cells and less stable than fresh donor blood.

Valeri et al (1970) reported survival measurements in 29 patients with erythrocyte mass deficit who required therapeutic transfusions. Eleven had neoplastic disorders, 15 were being treated for traumatic injuries, 2 had renal insufficiently and one had a brain injury and gastro-intestinal bleeding. No patients experienced chills, fever, urticaria, chest or back pain. Haemoglobinaemia and haemoglobinuria was seen in two patients who received at least 3 units of Huggins preserved cells which were less than 2 years old and had been washed by low ionic cytoagglomeration. Levels of plasma haemoglobin were greater than calculated as attributable to the total amount of infused supernatant haemoglobin. In both cases gross haemoglobinuria disappeared within 6 hours and repeat compatibility tests were negative. Later one of these patients who still required transfusion before surgery, was given three further units of Huggins stored blood recovered by the continuous centrifugation wash cycle using electrolyte solutions. He showed no ill effects and his plasma haemoglobin was as calculated. In neither of these two haemoglobinaemic patients was there any cardio-respiratory symptoms nor adverse effects on renal function. Urine output remained normal and blood urea nitrogen and creatine concentrations and the ability to

Concentrate urine were unimpaired. Nevertheless these authors decided to discontinue the therapeutic use of Huggins preserved cells that had been stored for over 1½ years and washed by cytoagglomeration.

Because of shortages of fresh donor blood and to avoid the problem of post transfusion hepatitis risk (Adashek and Adashek 1963; Robinson et al 1965; Walsh et al 1970) in such multiply transfused patients Valeri et al (1970) used 6 month old frozen blood from the Huggins method recovered by agglomeration in 11 surgical patients on cardio-pulmonary extracaporial circulation (p 54). Recovered units showed a mean supernatant haemoglobin of 400 mgm ± 57 mgm/100 ml on the day of recovery and 838 ± 261 mgm/100 ml 24 hours later immediately prior to operation. Supernatant potassium was 13.2 ± 2.3 mgm/l on recovery and 25.3 ± 3.5 24 hours later just before infusion. Plasma haemoglobin was higher in patients receiving frozen cells although haemoglobinuria occurred in both groups of patients. However there was no evidence of renal impairment in any group as measured by blood urea or creatinine, and coagulation measurements of platelet counts, partial thromboplastic times and fibrinogen levels remained satisfactory. In their Table 3 however one can see that haemoglobinaemia and haemoglobinuria was greater in the patients receiving frozen cells. Plasma haemoglobin in the frozen cell group 24 hour after operation showed an increase of 127.9 ± 85.7 mgm/100 ml compared to 106.2 ± 46 mgm/100 ml in the non frozen cell group. Urine haemoglobin showed 141.7 ± 26.3 mgm/100 ml in the non frozen blood recipients. Over the next 3 days post operatively, the urinary haemoglobin

was 149.2 ± 225.6 mgm/100 ml in the frozen cell group and 33.9 ± 42.2 mgm/100 ml in the non frozen cell group ($P < 0.05$).

Rudear et al (1970) reported Russian experienced with the use of frozen cells recovered from liquid nitrogen and giving to 12 patients undergoing heart surgery whilst on extracorporcel by-pass. Each patient received up to 2 litres of recovered cells in procedures lasting 41-291 minutes at a pump rate of 2.8 - 4.6 litres/minute. No adverse effects were found and they stated that in no operation was there an evidence of haemolysis due to mechanical trauma.

Tullis et al (1971) describing the benefits of having developed a disposable plastic bowl for the continuous centrifugal wash system reported that cells recovered had been transfused into 15 volunteer normal subjects and 42 patients. No adverse effects were found and patients circulating red cell mass rose consistent with transfusion replacement with standard non frozen ACD blood. Plasma haemoglobin before transfusion was 5 - 17 mgm/100 ml and 24 hours post transfusion 5 - 32 mgm/100 ml. Haptoglobin levels paralleled these in that pre-transfusion levels averaged 209.9 mgm/100 ml and post transfusion 169.8 mgm/100 ml. Average haematocrit after two units of blood rose from 30.6 to 34.4 vol/vol. These figures all reinforce the good recoveries and yields of frozen cells which these authors obtained. They found an average recovery of 89.1% and a post transfusion survival of 91.0% with at least 81.16% of these cells circulating in the recipient. (Allowing for cell losses in the flow tubes and residues left in the bowl and freeze thaw injury of, in total, 10.8%). The decrease in haptoglobin is equivalent to a mean loss of

less than 10 ml of frozen cells whose volume varied from 500 ml to 2,500 ml per recipient.

Bryant and Wallace (1974) in reporting their experience with frozen blood in a small hospital considered that the optimal inventory control and high levels of DPG and ATP were an advantage and there was no doubt that the use of frozen cells markedly reduced the incidence of febrile and hypersensitivity reactions as well as reducing the incidence of hepatitis and permitting auto transfusion and stockpiling of rare cells. They used over 3000 units of frozen cells with no adverse effect in any patient.

The presence of histocompatibility antigens in transfused blood is possibly generally undesirable but poses a particular threat to the dialysis patient awaiting kidney transplant. Significant reductions in white blood cells are difficult to achieve by conventional methods of washing or by filtration and numerous methods exist. Comparative studies have been done to compare the various methods one with the other and although it is certain that frozen cells gives the best results in terms of leucocytes in the residual cells transfused after recovery it would seem appropriate to outline some of these other studies for comparison.

Recognition that white cell antibodies were responsible for non haemolytic febrile transfusion reactions stems from the work of Dean in 1926 who reported that compatible red blood cell transfusions could result in severe, almost fatal reactions when leucocyte incompatibilities existed. He advised direct matching of white blood cells and plasma as an additional safeguard prior to transfusion. Today it is recognised that in addition to causing febrile transfusion reactions (Payne and Rolfs 1960; Chaplin et al 1959) the antibodies to the HLA antigens on leucocytes and platelets can jeopardize the survival of an organ transplant (Kissmeyer-Neilsen et al 1966) and compromise the response to component transfusions (Yankee et al 1969). It is also recorded that in special circumstances, the transfusion of viable lymphocytes may result in graft versus host reaction (Hong et al 1968; Polesky et al 1970). Kantor and Johnson (1970) reviewed the transmission of

cytomegalovirus in leucocytes and suggested a conservative approach using leucocyte-free stored blood might be used when transfusing patients particularly susceptible to opportunistic infections'. Despite numerous technological advances, availability of typing reagents, and a better definition of the antigens on leucocytes and platelets, routine testing to detect and prevent HLA sensitisation is time consuming and not feasible for every case. As an alternative, numerous procedures have developed to prepare HLA antigen-poor blood. The usual methods result in a sacrifice of a significant proportion of red blood cells and have a significant contamination of viable white cells. During the early work with the Cohn fractionation for deglycerolizing frozen thawed cells, Tullis (1958) noticed that recovered cells on transfusion resulted in less pyrogenic and urticarial reactions. Deglycerolised cells suspended in albumin did not result in reactions on patients who had had previous chills and malaise after standard ACD whole blood. Tullis and Lionetti (1966) reported a double blind study in three Boston Hospitals using patients known to have such reactions to whole blood. Their blood requirements were met by random allocation from group specific pools containing coded units of standard ACD blood and thawed deglycerolized cells in albumin solution. Neither the transfusionist nor the recipients were aware of the source of the units administered. Sixty seven units of deglycerolised red cells in albumin were administered to 12 recipients without pyrogenic reactions, 76 units of standard ACD blood given to the same group of 12 recipients produced 37 pyrogenic reactions.

Perrault et al (1967) recorded marked reduction in severely allergic transfusion reactions by using recovered frozen blood for patients who were not treatable without reactions occurring even to buffy coat poor and saline washed ACD blood.

It is well established that the intensity of reactions correlate with the number of incompatible cells transfused (Brittingham and Chaplin 1957; Perkins et al 1966) and is dose related in this sense. Not only granulocytes but lymphocytes and platelets can be responsible for these reactions. The HLA antigens are present on those cells as well as all nucleated cells in the body and hence their interest for the organ transplant immunologist. Small amounts of HLA antigen are present in plasma (Van Rood et al 1970) and some say perhaps even on the surface of red cells (Morton et al 1969) but these of course provide minimal amounts compared to the leucocytes and platelets. If antibodies to HLA are present as a result of previous antigenic stimulation they can be associated with hyperacute rejection of transplanted kidneys (Patel and Terasaki 1969, Braun et al 1972) It has been suggested that as many as 40% of patients awaiting kidney transplantation may be already immunised to HLA antigens primarily due to repeated transfusions of packed cells (Perkins et al 1973). Although efforts are made to keep the transfusions to dialysis patients to a minimum, they still occur and it is therefore considered advisable to prevent this stimulation if possible (Perkins). No one yet knows the exact minimum dose of immunogen required to stimulate a response to foreign HLA material so that the desirable end point of leucocyte

preparations is not predictable (Miller et al 1973; Polesky et al 1973). Clearly it must be a variable and sliding factor based on numerous donor differences, recipient differences and other treatments present and in the past. Engelfriet et al (1975) have established some useful data in white cell immunisation of rabbits. Specific granulocyte leucoagglutinin responses were demonstrative with the use of 0.5×10^6 leucocytes. Lymphocytotoxic antibody response needs doses of immunogen of the order of 1×10^6 cells on more than three monthly injections. Similar antibodies could be induced in rabbits given concentrated resuspension medium removed from processed frozen blood.

As well as purely removing the countable leucocytes and platelets, one has therefore to consider the possibility of whether the remaining cells are dead or viable, whether they can act as immunogens or whether they are modified and effectively silenced by the process for their removal, whether the presence of white cell debris and released leucocyte content of HLA material in the cell suspension medium is also capable of induction of an antibody response.

Meryman and Hornblower (1973) in considering the leucocyte depletion in frozen blood found that leucocytes are damaged by both the glycerolizing and the freezing. The removal of the cells does not appear to be the result of washing them out of the red cell suspension but rather from their aggregation into a mucous like mass that remains at the end of all washing systems (p 45). They showed that microscopically this consists of nuclei and white cell debris. In addition and more importantly since they can

get into a patient, many of the residual cells appear damaged suggesting that they may be non-viable. Schechter et al (1972) have investigated thymidine uptake and the appearance of atypical lymphocytes in transfused patients. They demonstrated that patients received unfrozen fresh or stored bank blood showed a marked response while patients receiving as many as four units of frozen cells recovered by automated bowl washing centrifuge showed no lymphocyte reaction. This suggests to these authors that even though a number of recognisable lymphocytes may remain, the glycerolizing and freeze-thawing have altered their antigenic qualities, a point also made by Polesky et al (1973). An even more interesting experiment concerns the freeze-thaw processing by a glycerolized method of separated donor leucocytes. After defrosting and wash recovery 6% of the original leucocytes were present and intact. Of these 99% were lymphocytes and 50% excluded trypan blue. None of the cells responded to stimulation by phytohaemagglutinin nor was a mixed lymphocyte reaction elicited nor was there any significant uptake of tritiated thymidine. These findings further suggest that although there may be some viable cells present, the freeze-thaw experience may alter their immunological characterisation by alteration of cell surface components. Surface changes on cells induced by freeze-thaw have been cited by Meryman and Hornblower of Robinson's work. Helgeson et al (1972) suggested that white cell fragments in frozen washed preparations are antigenic. Similar recovery figures were reported by Crowley et al (1973) in that 8% of leucocytes remained after freeze-thaw and washing and 2.6% of platelets

with 70% of the leucocytes being mononuclear cells some of which were viable. HLA antibody absorption studies further demonstrated that there was antigenic material present in the washed units which was not associated with intact leucocytes and which could be removed by micropore filtration. Goldfinger et al (1973) found automated bowl washing to be inferior to manual washing of cells which removed 2/3 to 3/4 of the initial white cells - enough to satisfy the clinical requirements in patients having febrile reactions to unwashed blood. They commented on the overall inferiority of simple washing methods with the 95% depletions which could be achieved with nylon filtration or red cell freezing programmes (see also Tenczar 1973; Miller et al 1973; Mitchell 1976 for review).

Polensky et al (1973 a) noted that of the various freeze-thaw recovery methods the most inferior was the cytoagglomeration method. This, of course, had been previously described by Valeri et al (1966) who showed that lymphocytes in the post freeze glycerolised state would agglomerate and pack with the red cells since they have at this stage the same size and density (vol.l.).

In this latter consideration it is valuable to examine the data for simple saline washing of red cells as a means of leuko depletion described by Tenczar (1973). Many clinicians believe that leucocytes are removed by saline washing and insist upon receiving only washed cells for patients experiencing reactions to 'unwashed' cells. Not only were leucocytes not removed in the washing but there was an increased loss of red cells in saline washed cells such that additional transfusions may be necessary to provide the desired clinical dose of red cells. Such extra transfusions might therefore mean the patients receiving

a larger cumulative dose of leucocytes with a broader spectrum of antigens derived from numerous allogenic donors. This is the reason that it has always been difficult to truly quantify and compare different methods of red cell 'purification' since one must remember that valuable quantities of red cells may have to be sacrificed in order to reduce the overall leucocyte load. Some workers have seen this difficulty and made an honest attempt to express the white cell count as a function of the residual red count, haemoglobin or haematocrit of the cells actually being transfused into the patient (Halterman et al 1972; Tenczar 1973). Such quantitative considerations are clearly necessary to improve the preparation and utilization of leucocyte-poor red blood cells (Perkins et al 1973).

Opelz (1974) has recently suggested that blood transfused may produce an antibody response which enhances graft rejection but most other authorities consider that the majority of evidence suggests that the formation of humoral antibody is often associated with hyperacute graft rejection (Jeannet et al 1970; Pierce et al 1971; Miller et al 1973; Tenczar 1973; Engelfriet et al 1975).

Suarez-Ch and Jonasson (1972) studied occurrence of HLA antibodies as determined by the lymphocytotoxicity method at monthly intervals in 135 patients on regular dialysis. Most of the patients had received whole blood or packed red cells prior to entering the dialysis programme. Only leucocyte-poor blood was used for transfusion following the start of regular dialysis. 23% of the men and 45% of the women (overall 32%) patients were sensitised to HLA antigen. Seven out of 19 patients were sensitised after

receiving only leucocyte-poor blood during a two year period. Pregnancy and previous blood transfusion were the most important factors but even leucocyte-poor blood will eventually cause sensitisation.

Goldfinger et al (1974) described how microaggregates can be removed from frozen cells by saline washing. These microaggregates are composed of white cell and platelet debris and increase in quantity with the age of donor blood stored in conventional banking. Such debris might be harmful in causing pulmonary intravascular occlusion as microemboli and this has led to the development recently of specific microfilters to remove such material which can pass through standard blood filters of 170 micron pore size. In frozen cell recoveries such debris occurs as a gel-like mass consisting of leucocyte (DNA and platelet debris which is strongly Feulgen stain positive indicating large DNA or nuclear material content). This material has been seen frequently in frozen cell recoveries and behaves as a tenacious gel which often adheres to the wall of the plastic wash bag, transfusion bag, wash bowl or is caught in the standard blood transfusion filter (p41). Crowley and Valeri (1974) further showed the nature of this gelatinous debris in that washing of frozen cells with solutions containing desoxyribonuclease prevented the aggregation and gelation of the debris. Polymorphonuclear leucocytes were preferentially removed and of the remaining cells, 70% were viable lymphocytes. HLA antibody adsorption demonstrated that particulate antigenic material was present in the washed red blood cells which could be reduced by micropore filtration. Crawley and Valeri (1974 b) published figures concerning the white cells, platelets and

plasma proteins in washed recovered frozen cells from a -80°C high glycerol type bank. 96.6% of leucocytes and 93.9% of platelets were removed by serial batch washing or bowl washing centrifuge. Best results were obtained with donor blood over 1 week old on the day of freezing. The residual plasma protein in washed previously frozen red cells was extremely low of the order of 0.00030 gm/dl supernatant. These authors recommend frozen red cells to prevent reactions to plasma, febrile leukoagglutinin reactions, possibly graft versus host disease and cytomegalovirus infection because of the low amount of residual plasma and small number of intact leucocytes.

The necessity of using leucocyte-poor blood in patients awaiting renal transplant has been accepted by most authors but more recently some doubt has been expressed by former protagonists (Morris, Ting and Stocker 1968; Lancet 1975). In 43 patients who received primary renal grafts from cadaver donors there was no relationship between the number of transfusions and the early course of the graft as judged by rejection crises. Of the 29 pregraft sera examined, three had lymphocytotoxic antibodies and these patients had a stormy early rejection course. Blood transfusion, they considered, may cause immunological unresponsiveness in renal allograft recipients due either to immunologic tolerance or enhancement. If so, tolerance, a state of specific unresponsiveness producted by treatment with antigens seemed the most reasonable explanation. Sengar, Rashid and Morris (1974) examined plasma samples from patients with chronic uraemia, renal allograft recipients, pregnant women and specifically sensitised recipients shown to contain a factor blocking

mixed lymphocyte reactions (MLC;B). This activity is concentrated in the non complement fixing IgG fraction of serum. Blood transfusion within 6 months preceding testing was significantly and directly correlated with the synthesis and persistence of MLC;B in the plasma of haemodialysis individuals. No association was noted in renal allograft recipients. The development of MLC;B seemed related with the formation of lymphocytotoxins but not with haemagglutinins. MLC;B was also demonstrated in the IgG fractions of serum obtained from chronic uraemic patients on dialysis and patients maintaining renal allografts. These authors believe that MLC;B may play a minor role in the acceptance of renal allografts in man. The MLC test should enable renal transplantation typing to distinguish these potential recipients who may be responders and have made antibodies to leucocytes. In other words, one should encourage the use of leucocyte-rich blood in transfusion to these patients in order to stimulate the production of leucocyte antibodies and MLC;B in a few of them. This will allow detection of the responders who on MLC testing may react by inhibiting the test thus identifying those potential recipient-donor combinations which may be advantageous. Nevertheless Callender (1974) reviewing 185 renal allotransplants noted 32 recipients had HLA antibodies and a negative anti-donor cross-match. These patients were compared with a carefully matched group of recipients who had no anti-HLA and to the total transplant group. No statistically significant difference occurred in allograft survival in any group. Myburgh et al (1974) noted that patients with antibodies to more than 70% of their lymphocyte test panel lost their grafts

(first cadaver). Sixty-one out of 114 patients were presensitised and had graft survival of 62% at 1 year and 53% at 2 - 5 years. In a non sensitised group of 50 patients, 1, 2 and 4 year graft survival was 86%, 83% and 79% respectively. Mean transfusion requirements while on haemodialysis were similar in non sensitised patients and those sensitised to less than half of the panel of test lymphocytes. Mean transfusion requirements were almost double (6 units) in patients sensitised to over 70% of the lymphocyte test panel. These authors emphasize that in their hands the graft survival in a majority of presensitised patients was good and different from what would be expected based on other reports in the literature (Terasaki, Kreisler and MacKay 1971).

Myburgh et al (1974 b), tried to clarify the situation. Assays for cellular (cell mediated lymphocytes CML) and humoral (antibody dependent lymphocytotoxicity and complement dependent lympholysis) evidence of graft specific pre-sensitisation were reported in 17 patients. In 4 of these serial post transplant assays were correlated in detail with the clinical course. This revealed a complicated and fluctuating interplay between the cellular and humoral immune responses. They could not define any set pattern to this nor could they make a confident prediction of the clinical outcome on any individual patient. CML may be negative despite unequivocal serological evidence of pre-existing graft specific sensitisation and a positive CML may be associated with a satisfactory clinical outcome. An abrupt but transient anamnestic increase in graft-specific antibodies following transplantation was associated with a very satisfactory clinical outcome and suggests but does not formally prove the occurrence of an enhancement phenomenon

(see also Morris et al 1968).Mi[illegible]al et al,1975.)

Perkins (1974) reported to the 27th meeting of The American Association of Blood Banks that he had tried to establish if indeed leucocyte-poor blood or frozen cells were really effective in avoiding antibody production to leucocyte antigens. Of 296 dialysis patients 78 had developed antibodies due to previous pregnancies, transfusions or transplants prior to entry to the study. 42% had detectable cytotoxic antibodies to lymphocytes. Excluding all immunised patients, 73 subjects had monthly checks for antibodies made during the study. Twenty-two received leucocyte-poor red cells only and 18% got detectable antibodies. Twelve received only frozen cells and none got detectable antibodies. Thirty-six received whole blood or red cells rich in buffy coat and 36% were immunised. He considered that such evidence was encouraging and suggested that frozen cells did not immunise to white cells although frozen cells did contain some leucocytes and platelets (Perkins, Senegal and Howell 1973). Roberts, Bauer and Camp (1974) showed that deglycerolisation from high glycerol frozen cells removed 97% of the white cells by dilution and osmotic rupture due to cell permeability for glycerol being slow with consequent osmotic lysis in isotonic media (vol,l.) and in low glycerol frozen cells 97% of white cells were removed by recovery and also freeze-thaw damage. In corroboration and expansion of this, Crowley and Valeri (197[illegible]) showed that red blood cells preserved with high glycerol/60°C technique had 4 times more leucocytes per gram of haemoglobin than did washed red cells preserved with 20% (low glycerol)/-150°C (rapid freeze) techniques. Despite removal of 94.3 ± 0.7% of leucocytes in

frozen cells recovered, Crawley et al (1975), showed that the remaining cells were mainly mononuclear and could be shown to be at least 50% responsive to phytohaemagglutinin indicating that some potentially immunocompetent cells remain. They considered that in the immunosuppressed recipient such cells could elicit a graft versus host reaction (GVH). Chaplin (1974) recorded personal communications from Rowe and Huggins that a reduction in leucocyte antibody reduction was demonstrable. Briggs in our own transplant patients together with Dick have recently assured me that there is at least a third reduction in the frequency of antibodies to white cells in frozen cell recipients (personal communication 1976). Oh et al (1972) reported a progessive conversation of sera from negative to positive for cytotoxic antibodies so that 60% of their patients receiving conventional blood transfusions were positive by the end of an 18 month period. In contrast and with smaller and less frequent transfusions, Manzler and Nathan (1974) found no evidence to support this.

Since the extent to which transfused red blood cells must be depleted of leucocytes before cytotoxic antibody formation and consequent diminished transplant survival is unknown (Opelz et al 1973) the clinical significance of the decreased number of leucocytes in red cell units freeze preserved by low glycerol remains to be determined. Mean leucocyte count in such methods was 61.1 x 10^6/gramHb before freezing, 35.0 x 10^6/gm Hb after thawing and 1.5 x 10^6 /gm Hb after processing, washing and preparation (see also Efficiency of process section, vol,1., Crawley et al 1973).

Opelz, Mickey and Terasaki (1973) studied data from 11 haemodialysis centres in Los Angeles. More than 90% of the

transfusions were given as leucocyte poor blood and the remainder as whole blood. Of 224 patients who had not been previously transfused 9.8% had lymphocytotoxic antibodies. 4% of the non transfused males had lymphocytotoxic antibodies probably due to "errors in data collection", and non specific cross reactions. A direct relationship was found between the development of lymphocytotoxic antibodies and the number of units of blood received. However approximately one half of the patients who received 26-30 transfusions did not develop lymphocytotoxins. Only 39% of 119 patients with known lymphocytotoxic antibodies remained consistently antibody positive. Of those who became cytotoxic antibody (LC) negative, some remained negative while others became positive again. It is suggested that the absence of LC antibody in such a high proportion of patients is induced by transfusion, rather than being due to selection of non responders.

Opelz et al (1973) further reported that in transfused patients who have not formed cytotoxic antibodies, kidney graft survival is significantly better than in non transfused patients. Engelfriet et al (1975) in assessing this work stated that Opelz and co-workers were nevertheless of the opinion that at present it is better to prevent immunisation in future recipients of kidney transplants. They considered the main reason for this being that cytotoxic antibodies of such broad specificity may be formed that the recipient becomes untransplantable. Also, weak cytotoxic or non cytotoxic antibodies may be formed that are raised in the pregraft cross-match with donor lymphocytes and which may still be capable of endangering the graft. Similarly immunisation

of patients waiting bone marrow transplantation or who may require later platelet infusions would best be avoided.

Weiden et al (1975) have recently written of the effects of time on sensitization to haemopoietic grafts by preceding blood transfusion. Although this work was in dogs whose immune and HLA system is different from mass there is sufficient interest to indicate that in human bone marrow transplantation caution should be exercised in the use of pre-transplant blood transfusion.

PART VI

CLINICAL STUDIES IN THE PRESENT WORK

"There is much in this book that is new
and much that is true; unfortunately
nothing that is new is true and
nothing that is true is new".

Attributed A.E. Housman (Nature 240,
323 1972).

CLINICAL STUDIES IN THE PRESENT WORK - THE THREE PHASES OF DEVELOPMENT

The approach to treatment of patients in the West of Scotland is in three phases. Phase 1 was associated with development of the optimal method for our conditions and a feasibility study. Phase 2 concerned the gradual build up of experience and confidence in patient care and phase 3 concerned the establishment of additional facilities in Glasgow hospitals (Fig.6/1).

Table 6/1shows the present capability of the frozen cell programme and trace its development in terms of patient care in the years 1971-1975. The frozen cell bank was extended to have a small satellite bank at Glasgow Western Infirmary from February 1973. The figures for these transplant and renal dialysis recipients are shown separately. In February 1976 a similar bank was started at Glasgow Royal Infirmary.

Taken overall, over 400 cases have now received a total of just over 2,500 units of frozen cells. All units have been sterile.

In general all patients have taken the frozen recovered cells without difficulty. Post transfusion rises in haemoglobin have been satisfactory (p 60) and only one patient had some transient haemoglobinuria. This patient, a 14 year old female suffering from erythrogenesis imperfecta had had numerous transfusions of frozen cells as part of her management. In September 1975 cells were prepared in the standard manner and sent to the Sick Children's Hospital. The transfusion was given without difficulty but the following morning she passed some dark urine which was

Centre	Source/Diagnosis	New 1971		Repeat 1971		New 1972		Repeat 1972		New 1973		Repeat 1973		New 1974		Repeat 1974		New 1975		Repeat 1975	
		Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused	Cases	Units Transfused
B.T.S.	Renal Unit	5	15			38	110			32	106	12	46	27	98	12	61	21	179	37	205
	Factor VIII Inhibitor	1	4																		
	Erythrogenesis Imperfecta									1	5					1	14			1	15
	Thallasaemia Major					1	6					1	6								
	Congenital Hypoplastic Anaemia					1	14					1	28			1	24			1	25
	Aplastic Anaemia	1	8			4	62			1	4	2	31			1	27	3	27	1	28
	Haemolytic Anaemia					1	4														
	Sideroblastic Anaemia					1	8			1	11										
	Myeloid Leukaemia					2	55			1	6	2	10	1	16	1	12	1	4		
	Lymphatic Leukaemia									1	2			1	8	1	2				
	HLA Antibodies					2	10														
	Other Antibodies					2	3			1	3			1	4						
	Rh D Immunisation Scheme									18	10	3	1	14	6	21	12	40	32	72	21
	Miscellaneous	1	1			3	10			3	5			4	8	1	5	8	62	1	3
		8	28			55	282			59	152	21	122	48	140	39	157	73	294	113	297
Western Inf. Glasgow	Renal Unit									18	77	17	82	116	258	29	290	58	302	33	297

	TOTAL CASES	TOTAL UNITS TRANSFUSED
B.T.S.	243	1472
Western Infirm.	192	1306

Table 6/1. Low glycerol cell usage in West Scotland 1971-73.

shown to be due to transient haemoglobinuria. No casts or red cells were seen in the urine and urine output and subsequent renal function was normal. When findings of haemoglobinuria became known a full investigation of possible transfusion reaction was done. Rematching of the blood to the patients pre-transfusion serum revealed no mismatch and the bacteriological testing of the unit proved it to be sterile. Close questioning of processing staff revealed no unusual appearances of the unit at the time of preparation and supernatant fluids had been as predicted. Indeed other units had been prepared at the same time for another patient and these were used without any problem. The donor of the offending unit had given blood on two previous occasions within the previous year and was so far as is known is perfectly healthy. At the hospital it was revealed that the donor blood had been approximately 6 hours out of the blood bank refrigerator before it was transfused. There was no clear record of where it had been in the ward for these 6 hours. Additional spectrophotometric scans of pre and post transfusion specimens of recipients of frozen cells are shown in Appendix.

Cases of haemoglobinuria following transfusion of frozen blood are scarce in the literature. Valeri (1964, 1968) reported some 14 unfortunate experiences in early work due to resuspension of cells in outdated human albumin solutions. Patients developed marked but transient haemoglobinuria with no residual renal impairment (Tullis 1965). An extensive study in monkeys given frozen blood during shock therapy revealed some microscopic evidence of renal tubular damage but again this was transient (Thompson

et al 1965). Almond and Valeri using cytoagglomerated cells in the treatment of stable anaemic patients reported that the patients haemoglobin and haematocrit were comparable to control patients receiving non frozen blood. Non viable cells were removed via an extravascular mechanism. Higher plasma haemoglobin concentration and lower serum potassium were noted in recipients of deglycerolised blood but no adverse effects on renal function could be observed. Brandt et al (1951) showed no adverse effect of haemoglobin solutions on renal function in man. Indeed haemoglobin solutions have been advocated from time to time as volume expanders in hypovalaemia (Rabiner 1967, 1969). In these, of course, there is a difference from haemolysed blood in that all red cell stroma is removed and one has only a solution of purified haemoglobin free of cell debris. Haber et al (1968) reported the use of frozen cells in thalassaemic multiply transfused patients with no adverse effects. Huggins et al (1971) reported a large series of clinical uses of frozen cells as follows: open heart surgery 874, kidney transplant and dialysis 429, red cell antibodies 223, white cell antibodies 88, substitution for ACD packed cells 659 cases. No elective surgical operations were cancelled because of lack of blood, auto transfusion was possible, the risk of hepatitis was diminished and the universal donor concept was restored. Jones et al (1968) reported that their 50 patients showed satisfactory rises in haemoglobin and none had any adverse effect. They reported the use of frozen cells for exchange transfusion due to anti-U (Puno et al 1967), auto transfusion in Jehovah' witnesses and for the prevention of febrile and allergic

reactions to leucocytes and plasma. Haemoglobinuria is undesirable for other reasons - for example it can interfere with the proper investigation and interpretation of possible transfusion reactions (Pert, Schork and Moore 1962) since haptoglobins would be bound to haemoglobin and haemoglobinuria would suggest intravascular haemolysis. This effect is mostly seen in ABO incompatible transfusions and is rare. Also since only group O cells are used in the present study it should not materially influence whether or not haemoglobinaemia is due to this cause (a small rise can occur with rhesus incompatibility and other related incomplete antibodies - Chaplin 1950). Most cells are destroyed in the reticulo endothelial system. Chaplin and Cassell (1960) found the mean normal plasma haemoglobin in 25 normal subjects as: 0.31 mgm - a very low value.

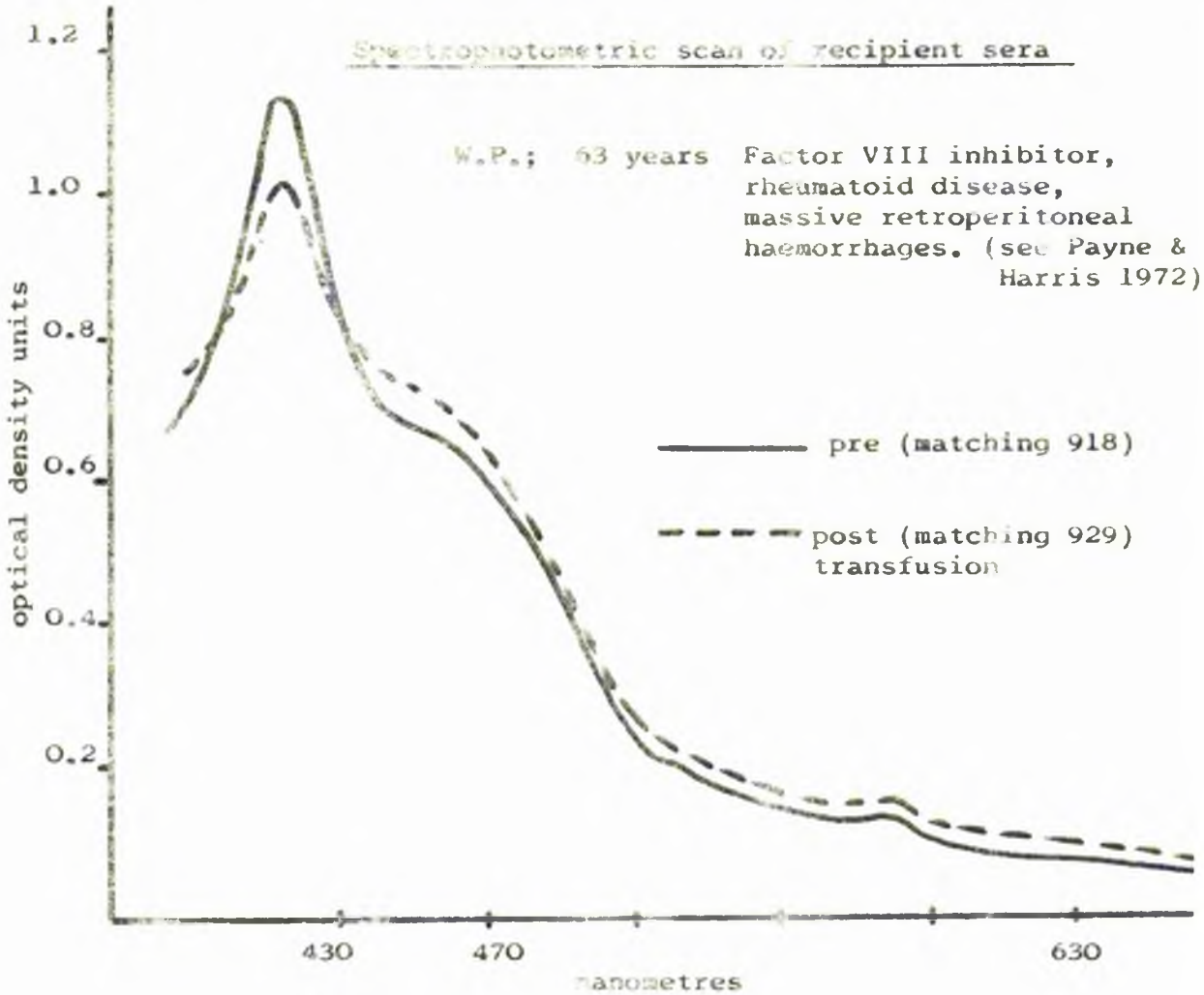
1 ml of red cells represents 360 mgm haemoglobin and a reasonable value for an adult's plasma volume is 3000 ml, then the rapid destruction of 1 ml of donor cells should raise the recipient's plasma haemoglobin by 12 mgm/100 ml.

In using full units of recovered cells stored for up to 5 days at $4^{o}C$ before transfusion Ketchel et al (1956) found recipient serum haemoglobins of 30-40 mgm/100 ml. When using blood stored for 1-2 weeks post recovery, the highest recipient serum haemoglobin was 85 mgm/100 ml. These all fell to normal within 24 hours after transfusion. The case shown in the 1972 Table of the present work refers to exchange transfusion in a serious case of haemolytic disease of the newborn. The sensitised mother had had no ante-natal care and was admitted just prior to delivery in a Glasgow hospital. At birth the infant was deeply jaundiced and it was strongly direct antiglobulin

positive due to a coating of maternal anti-e. Bilirubin was 24 mgm/100 ml and with no time available to screen for selected e negative donors a search of the frozen cell inventory produced a suitable group O R2R2 donor. The recovered cells were recovered and resuspended in 70 ml plasma protein solution (4.3%). After exchange transfusion the baby's bilirubin fell from 24 mgm/100 ml to 4 mgm/100 ml. It continued to thrive and when seen at the paediatric return clinic six weeks later, the haemoglobin was 12.2 g/dl. Since this incident the same patient has been delivered of another unaffected baby in 1975. Needless to say, on this occasion there was again little warning but the patient was admitted to hospital in early labour and kept there until delivered!

Miles et al (1968) reported the use of frozen blood for 44 patients with multiple red cell antibodies where it would be difficult to obtain sufficient units selected for such patients from liquid banks. These 44 patients received 129 successful transfusions of cells from a stable inventory of fully typed blood. This same approach has been used by Grove-Rasmussen and Huggins (1968, 1973) (see p 96).

Krijnen et al (1968) in their early experiences with the low glycerol intermediate freezing system found no adverse reaction to recovered cells in four patients. Haemoglobin (red cells) rose, there was no evidence of "hyperhaemolysis" and no adverse effect on renal function. Radiochromium labelling of infused frozen cells into three patients showed less than 1% of the label in the urine in 24 hours; plasma haptoglobin and bilirubin levels likewise showed no suggestion of significant red cell destruction in



	whole blood Hb g/dl	Plasma Hb mgm/dl	plasma haptoglobin mgm/dl
pre transfusion 15:11:71	5.5	13	108
post transfusion 2 units 16:11:71	7.0	1	100
pre transfusion 17:11:71	5.9	3.5	-
post transfusion 2 units 18:11:71	7.1	35	120

Urobilinogen absent

Haemoglobinuria absent

Jaundice absent

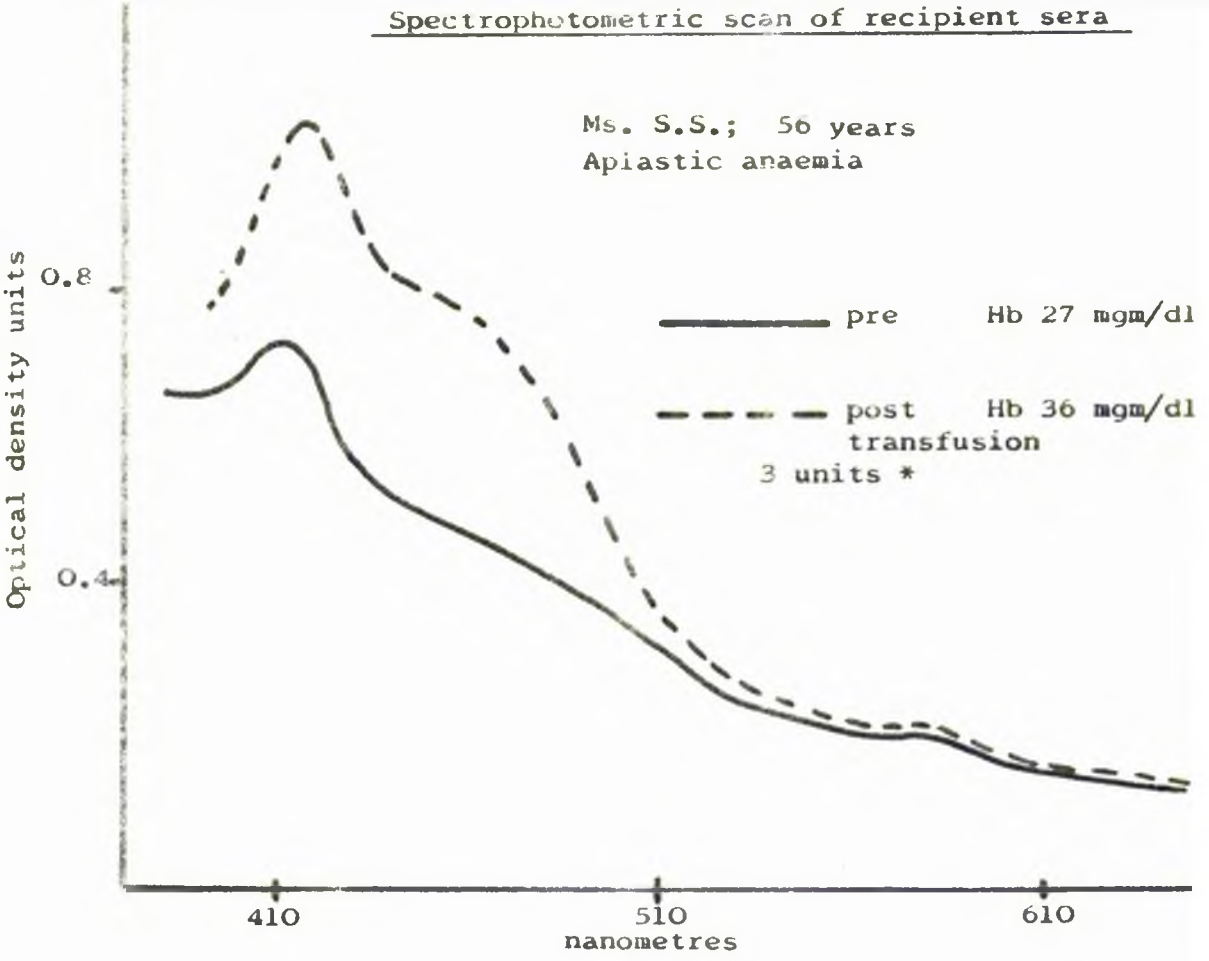
Factor VIII rose from less than 1% to 9% on 25:11:71

Fig 6/[illegible]

vivo (Laurell and Nyman 1957; Latham 1959; Langley et al 1962).

Numerous experiences are now available with the use of frozen blood in combat casualties (Moss et al 1968; Moss et al 1974).

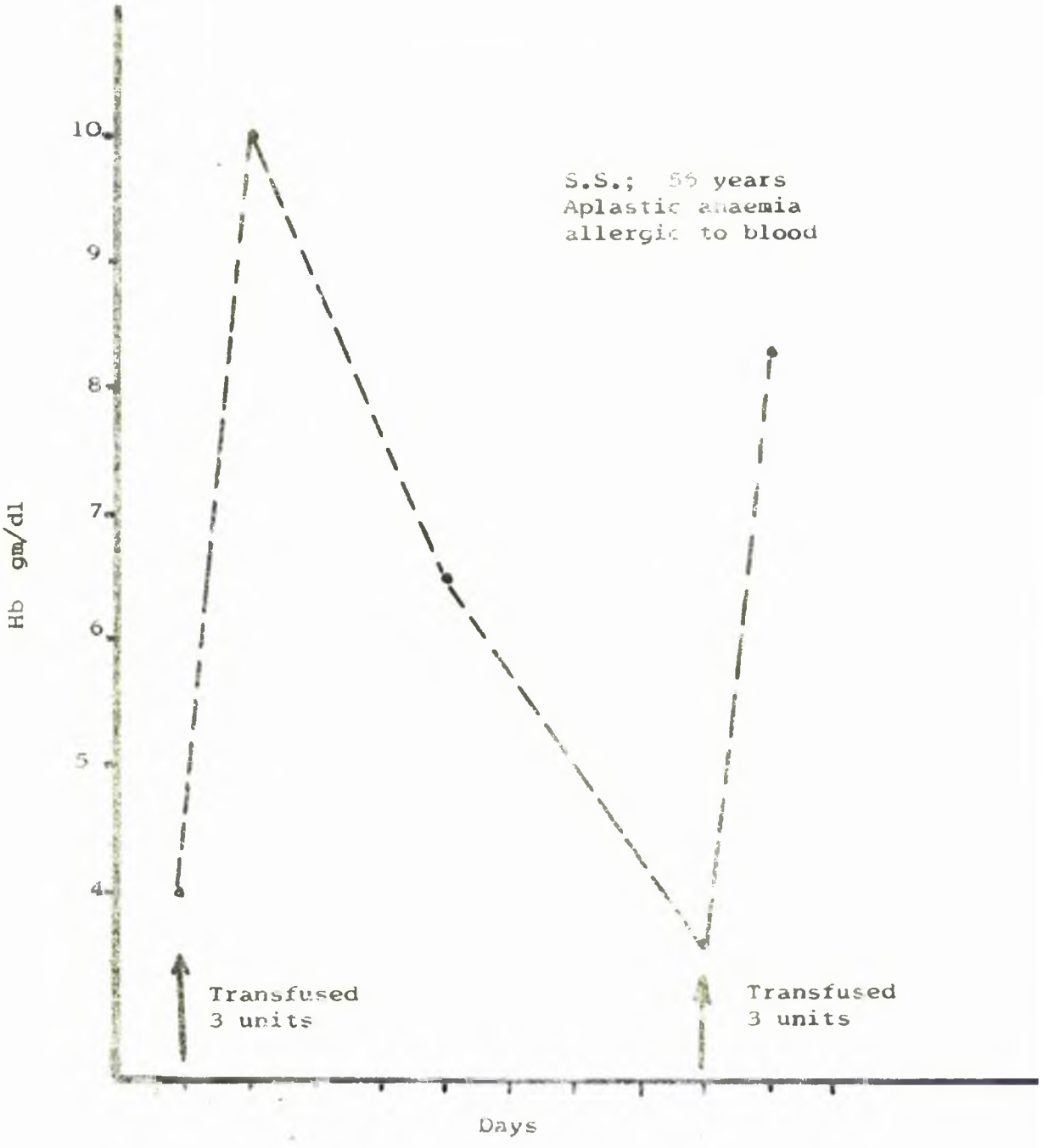
Button et al (1972) described a case of prevention of "anamnestic" response in a patient who had an inhibitor to anti-haemophilic factor. This was similar to the case reported by Payne and Harris (1972) who described a severely affected man with a large retro peritoneal haemorrhage due to factor VIII inhibitors who was intolerant of any fresh plasma or factor VIII. This man was one of the first ever frozen cell recipients in the West of Scotland. His condition of severe anaemic anoxia and hypotension rapidly improved after frozen cells. (Fig.62) This man continued to improve and survived to be treated with azothiaprim immuno suppression. His factor VIII inhibitor diminished and he is today alive and well with no symptoms, three years after the episode of his incipient demise. The spectrophotometric scan chart shows the appearance of the patient's plasma 24 hours before receiving two units of frozen cells on 15th November, 1971 and the appearances before receiving a further 2 units on 17th November. Oxyhaemoglobin is free in his plasma 24 hours after the first two units of frozen cells (measured in two specimens taken into sequestrene tubes and rapidly separated with minimal trauma). The absorption bands of oxyhaemoglobin are easily seen at 541 and 413 nanometres. No abnormal pigments are to be seen and there is no marked increase in free haemoglobin. Indeed reference to the clinical data shows that before transfusion his plasma



pre transfusion	whole blood Hb g /dl	Plasma Hb mgm/dl	Haptoglobin mgm/dl
8:11:71	4.0	-	-
post transfusion (3 units)			
9:11:71 *	10.0	36.0	520
10:11:71	-	32.8	544
12:11:71	6.4	0.6	520
15:11:71	-	0.0	-
pre transfusion			
16:11:71	3.6	4.6	492
post transfusion			
17:11:71	8.3	43.0	480

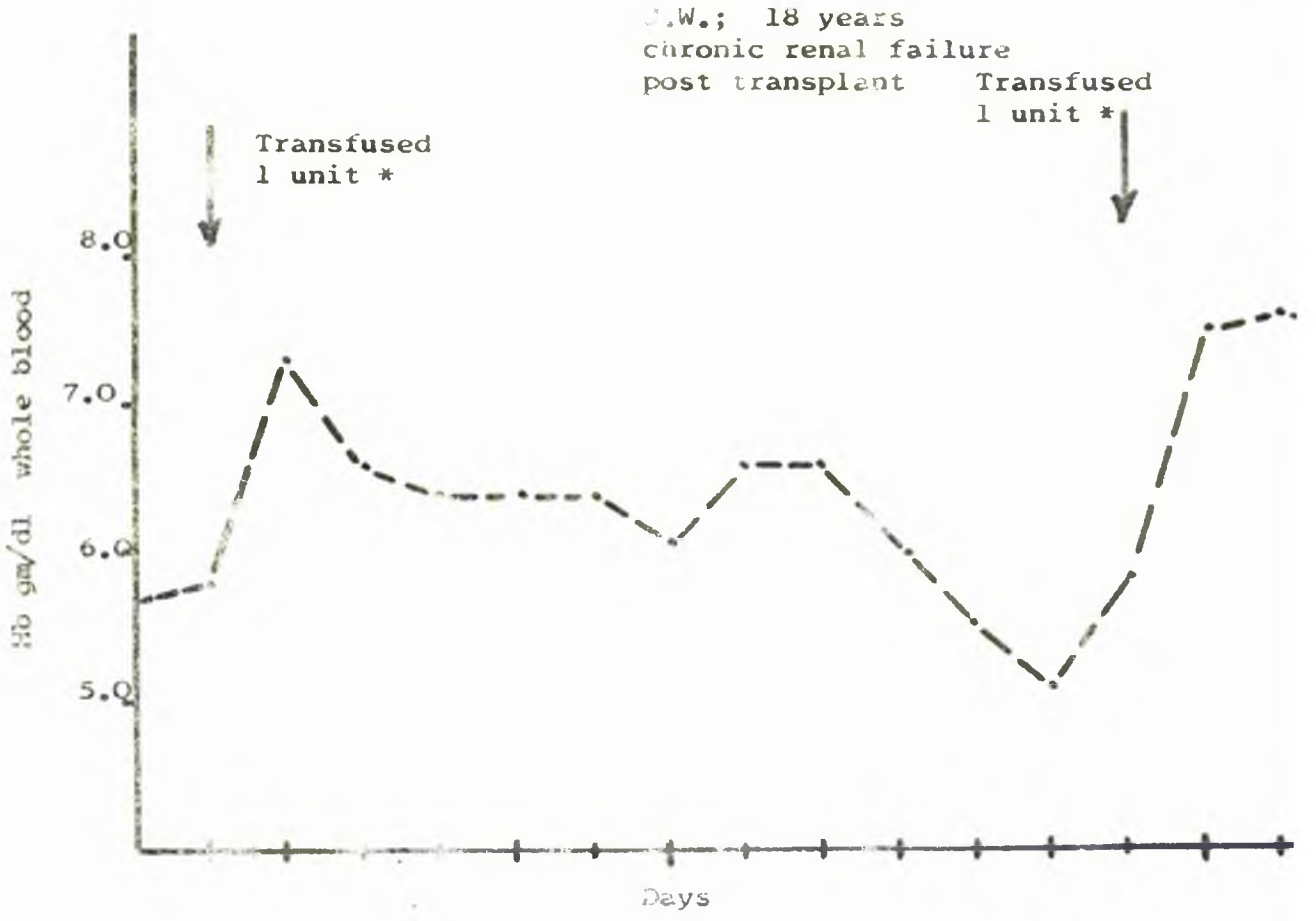
Haemoglobinuria	absent
Abnormal pigments	absent
Jaundice	absent
Adverse reactions	absent

Fig 6/3.



Response to frozen blood

Fig 5/4.



Response to frozen blood

* unit	Hb gm/dl	Hb gm/unit
25559	21.2	61.4
25570	19.6	58.8

Fig 6/5.

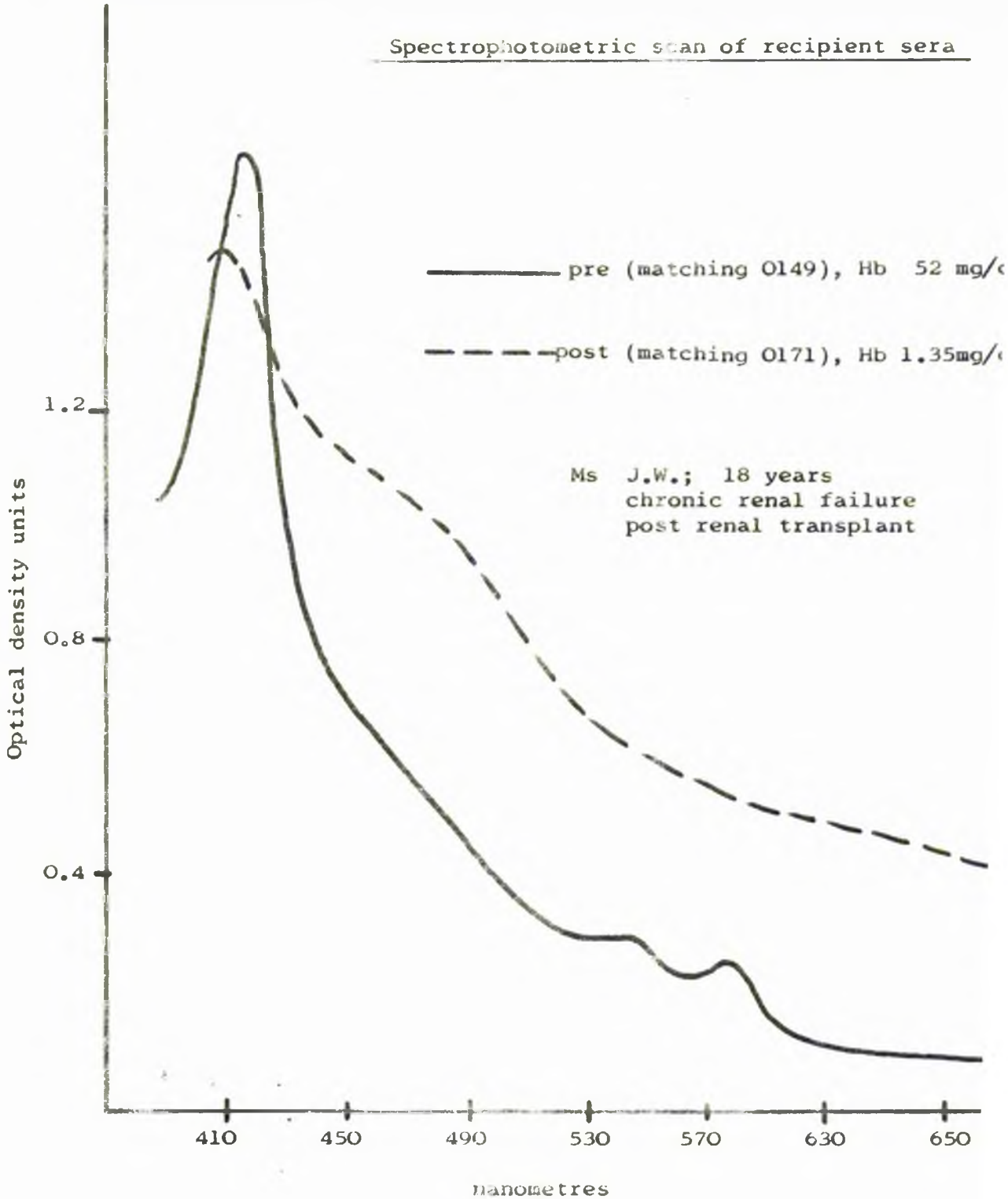
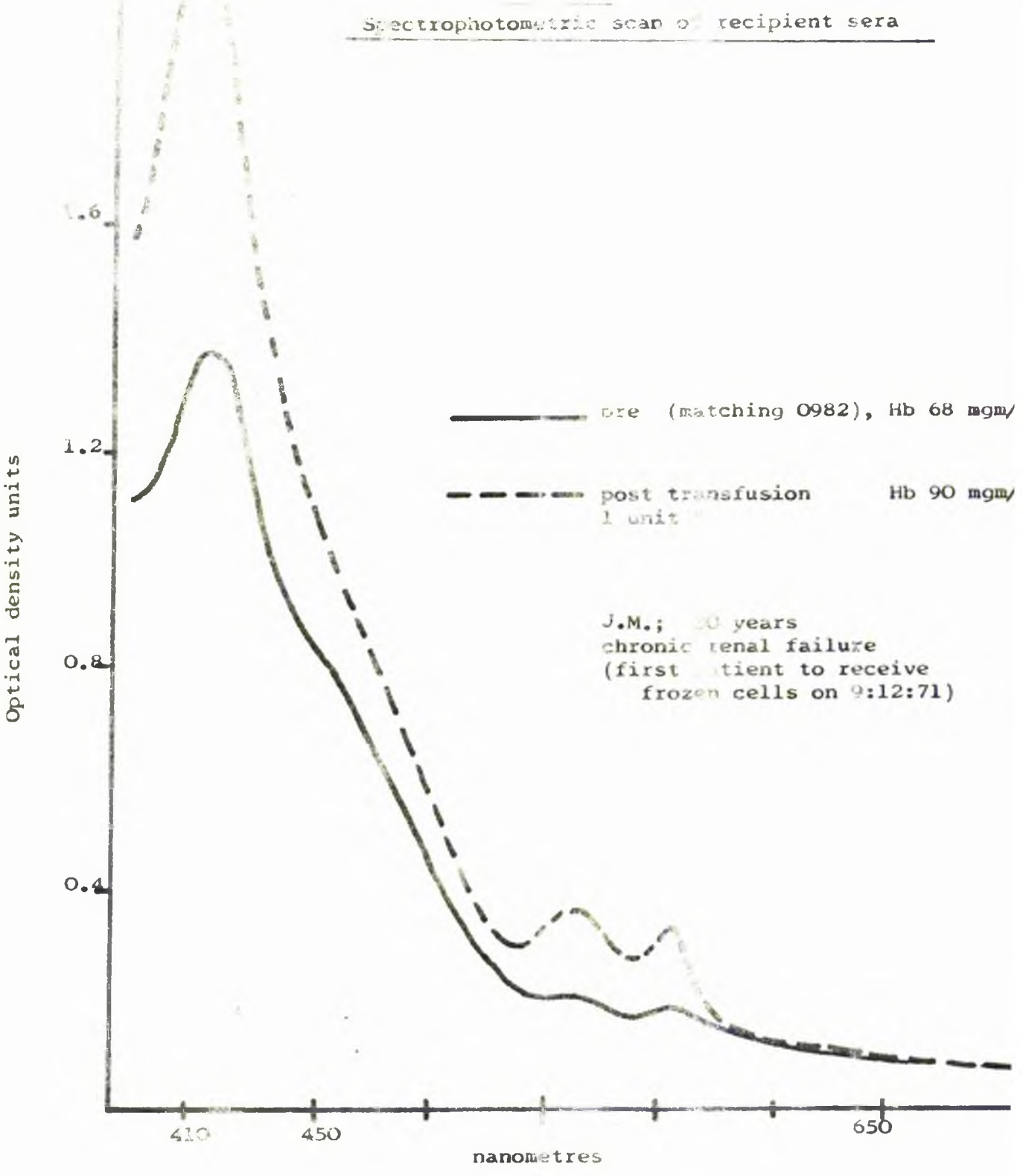

Spectrophotometric scan of recipient sera
pre (matching 0149), Hb 52 mg/
post (matching 0171), Hb 1.35mg/
Ms J.W.; 18 years
chronic renal failure
post renal transplant
Optical density units
1.2
0.8
0.4
410
450
490
530
570
630
650
nanometres

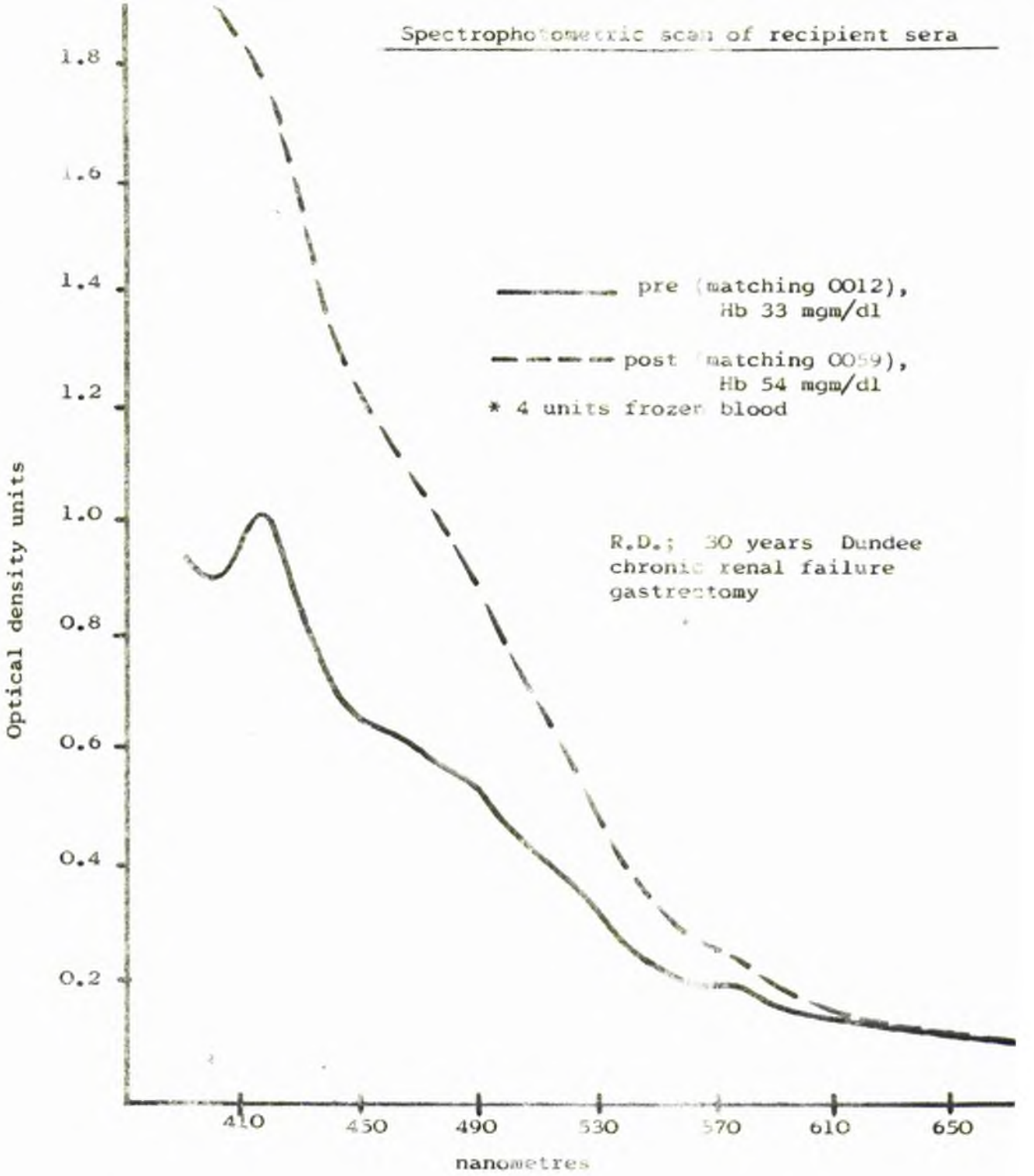
Fig 6/7.

Spectrophotometric scan of recipient sera



* unit	Hb gm/dl
51504	17.1

Fig 5/8.



Units	Hb gm/dl	Hb gm/unit
85996	10.1	29.29
85989	20.0	58.0
859	16.8	50.4
86021	13.1	35.57

Fig 6/9.

haemoglobin level was 12.0 mgm/100 ml and 18 hours later, following a 6 hour infusion of frozen cells, his plasma haemoglobin had in fact fallen to 1 mgm/100 ml.

Fig 6/3. shows the recorded findings in the first patient at Glasgow Royal Infirmary to receive frozen cells and the first ever to receive frozen cells from our bank (see p. 62). Spectrophotometric scans of the patient's serum separated for matching tests No's. 900, and 921 showed no abnormal pigments present and the Table shows the levels of free oxyhaemoglobin at the time intervals shown.

The first renal dialysis patient to receive frozen cells was a patient in Stobhill Hospital on 9th December 1975. Unfortunately no clinical appraisal was done other than the observation that the cells were tolerated without adverse effect. It was possible to obtain one sample from the patient 24 hour post transfusion and the plasma haemoglobin could be compared with the pre-transfusion sample, as shown in the spectrophotometric scan (Fig 6/5). Here one can see that there is only free oxyhaemoglobin present in both samples and there was no abnormal pigments. Measurement of this oxyhaemoglobin by Drabkin's method showed pre-transfusion 68 mgm/100 ml.

These findings would agree with other authors that (a) frozen recovered cells can be transfused without any difficulty, (b) satisfactory clinical responses are obtained, and (c) no abnormal pigments or renal impairments occur.

Since these early beginnings, of course, thousands of units have been transfused without any difficulty (see Table 6/1) and with good clinical response as measured

by the "no news is good news" philosophy.

We in the Regional Transfusion Centre have done everything possible to ensure that the frozen cells meet all of the desirable criteria laid down by others of (a) a sterile product delivered in the best possible condition to a patient which is free of leucocytes, platelets, cell debris, excessive free haemoglobin and which contains sufficient viable cells to make its infusion worthwhile.

As a result of these early studies a meeting was held at Glasgow Royal Infirmary on 25th November 1971. A copy of the minute of this meeting is enclosed as part of the clinical appendix of this thesis. As a result of this we introduced the regular use of frozen cells in Glasgow Dialysis Units (Stobhill, Western and Royal Infirmaries) and the author presented the first results to a meeting of the West of Scotland Blood Club on 24th May 1972. This Paper is included in the clinical appendix. As well as this, a preliminary contribution was delivered to the Society of Low Temperature Biology meeting on 29th October, 1971 dealing with early technical achievements. This is also included in the technical appendix.

Red cell survival studies in this work proved elusive and only two survival measurements on post thawed cells were made. I am grateful to Dr. J. Selwyn, Consultant Haematologist to quote his case of chronic anaemia who had a normal chromium survival when frozen red cells were labelled with radio-chromium. The cells were recovered at the Regional Transfusion Centre and a 50 ml sterile sub-sample sent in a sterile container with the donation to Dumfries where the sub-sample was labelled and given at the time of

E.J.; [illegible] years
Anaemia (p.k. deficiency)

Response to frozen blood

* Units	Hb gm/dl	Hb gm/unit
86000	18.6	59.94
25357	12.9	45.15
25353	12.0	38.40
86026	12.7	38.10

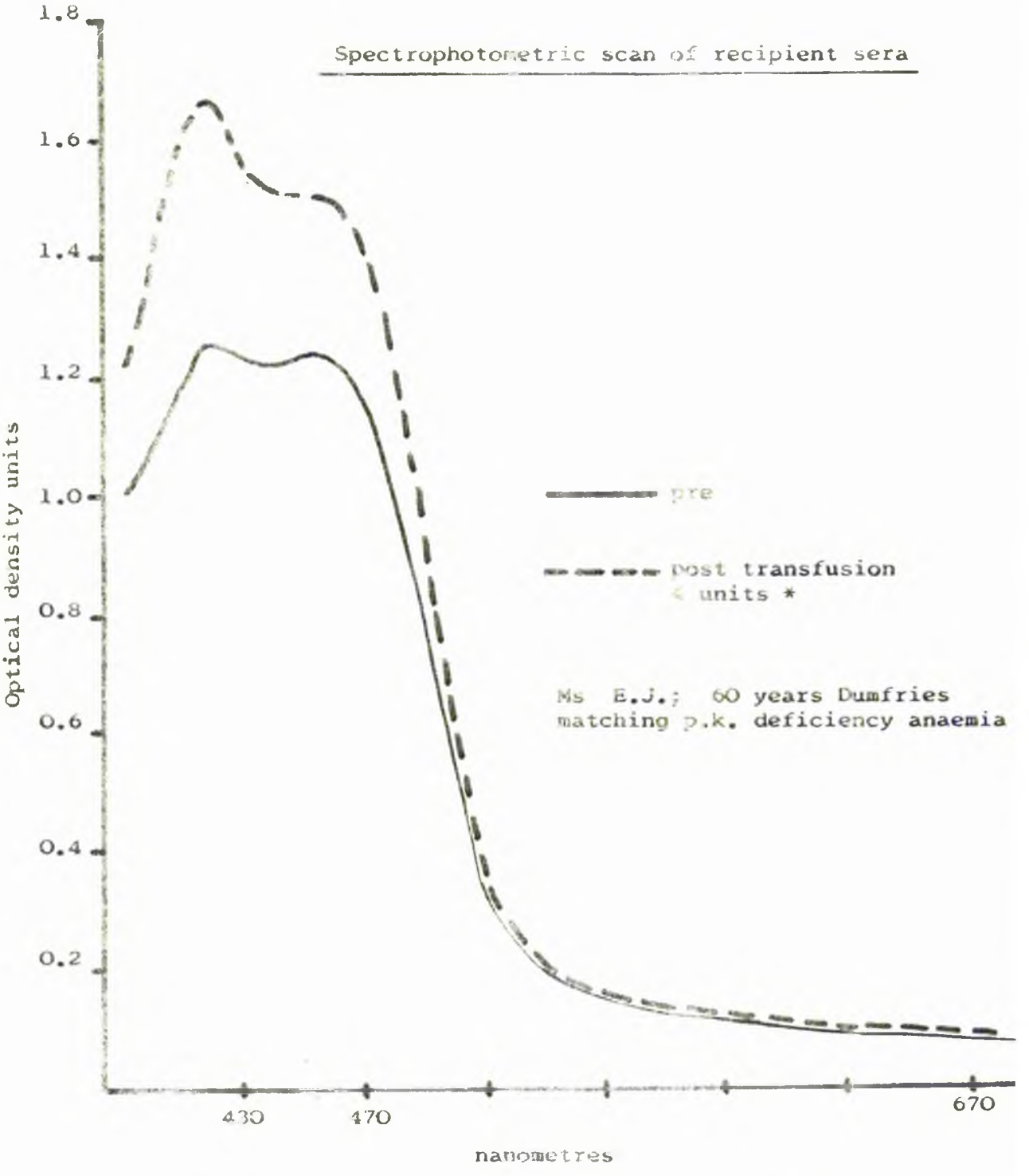
Fig 6/11.

Response to Frozen Blood

E.J. 60 years, Dumfries

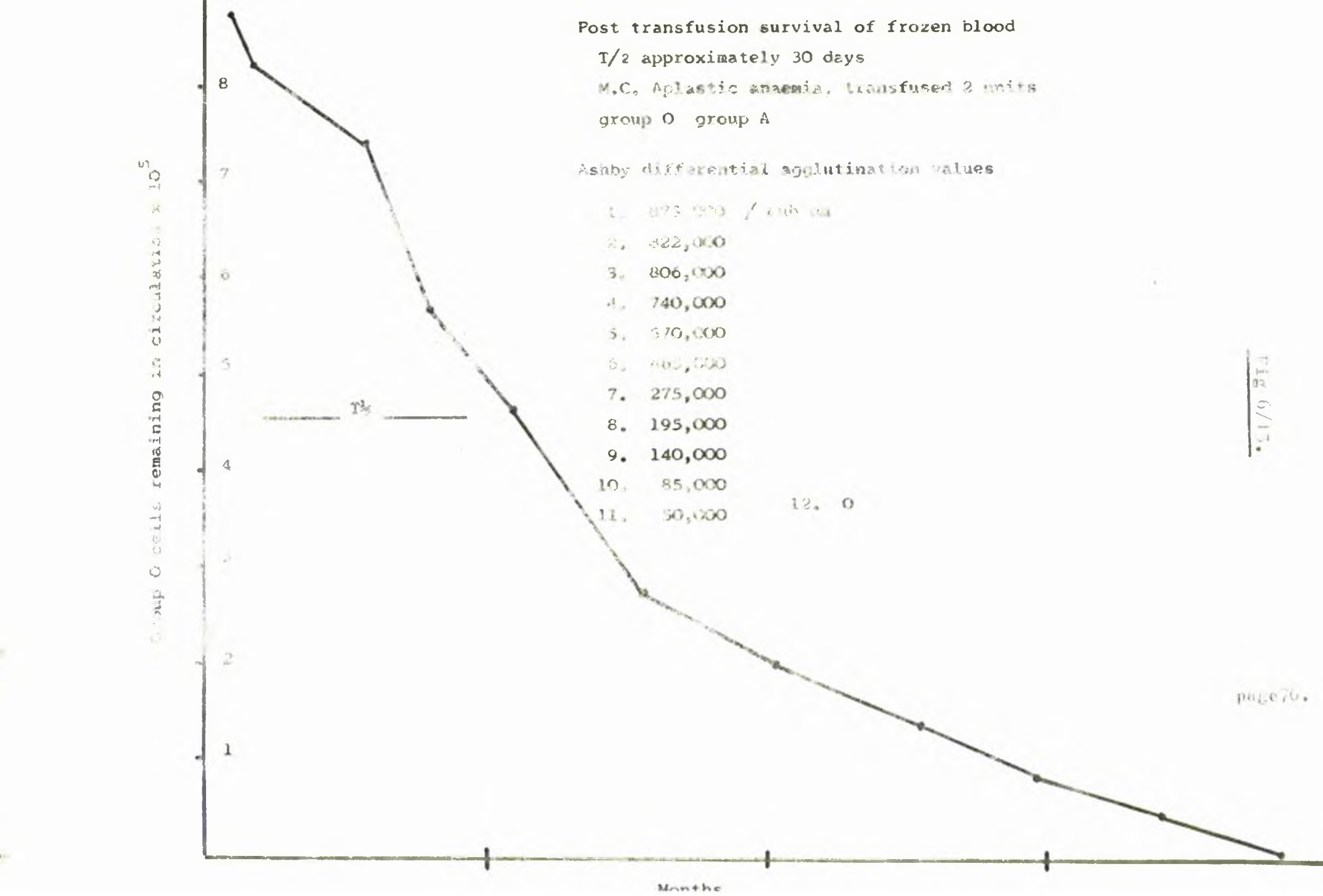
	Pre transfusion	Post transfusion (4 units)
Hb gm/dl	6.7	11.6
P.C.V. l/l	0.21	0.35
MCHC gm/dl	32.0	33.0
Urobilinogen	absent	absent
Haemoglobinuria	absent	absent
Adverse reaction	absent	absent
24 hour ^{51}chromium survival	Day 1 94% (normal)	
	Day 2 91.5% "	
	Day 3 87.0% "	

Fig 6/11.



unit	Hb gm/dl	Hb gm/unit
86006	18.6	59.94
25357	12.9	45.15
25353	12.0	38.4
86026	12.7	38.1

Fig 6/12.


Post transfusion survival of frozen blood
T/2 approximately 30 days
M.C. Aplastic anaemia, transfused 2 units
group O group A
Ashby differential agglutination values
1. 873 000 / cmm
2. 822,000
3. 806,000
4. 740,000
5. 570,000
6. 460,000
7. 275,000
8. 195,000
9. 140,000
10. 85,000
11. 50,000
12. O
T½
Group O cells remaining in circulation x 10^5
8
7
6
5
4
3
2
1
Months

the transfusion of the whole donation. Serial samples from the patient were then counted for radioactivity and a plot of the radioactive cell population remaining was made. From this, the decline in red cells was similar to that of non-frozen cells. Plasmahaemoglobin studies are shown in the Fig. before and after transfusion.

Using a differential agglutination technique of Ashby, Dr. N.P. Lucie of Glasgow Western Infirmary counted the number of surviving group O donor cells, recovered from frozen bank at the Regional Centre, in a group A recipient. Sequestrene samples of peripheral blood at intervals after transfusion were incubated with an excess of anti-A to remove all group A cells (Greendyke et al 1965). Thereafter the surviving group O donor cells were counted in a Coulter model S electronic particle counter with the result shown in the Fig.6/13In this it is seen that the frozen cells have a survival in the circulation of a stable anaemic patient of at least 109 days.

These results are encouraging and certainly agree with the situation reported for frozen cell survival in the literature. Recently it was decided to make another attempt at making observations on in vivo survival of recovered frozen cells. It is known that considerable variations exist between donors in the same recipient (Mitchell 1976). To avoid any difficulty of having to use the same recipient on more than one occasion, it has been decided to label normal cells collected freshly from a donor and to inject these with a similar quantity of frozen recovered cells from the same donor labelled with a different radioisotope. In this way it is hoped that

we will have a direct comparison of conventionally stored cells and frozen cells without variation between unrelated donors. In this sense the donor is acting as his own control. One sample of cells will be labelled with Tritiated (^{3}H) Di-isofluorophosphate (DFP) and the other with radio-chromium. Preliminary studies have been made at the Regional Transfusion Centre on the radioactive DFP label to ensure that it is non-pyrogenic and the outcome of these experiments is awaited with considerable interest. Double labelling techniques have been used by other authors (Szymanski et al 1961; Prins et al 1965; Szymanski et al 1969; Szymanski and Valeri 1968, 1970; Szymanski, Valeri and Emerson 1971; Szymanski et al 1971, 1973).

Following the introduction of the frozen cell bank at Glasgow Western Infirmary on 1st February 1972 that hospital took over the preparation of frozen cells supplied by the Regional Transfusion Service. After some initial set back whilst technical staff were becoming familiar with the new techniques employed, cells have been prepared without difficulty. Initially cells were prepared on demand but this led to delays during the night and technicians on call who had been taught the techniques had difficulty in meeting the strict time-table requirements of pre-transplant preparations at short notice. At one point early in this transition period the Regional Centre offered to take back some of the load of day-time cases and pre-arranged blood transfusions for patients on intermittent but predictable haemodialysis. Dr. M. Hutchison, Consultant Haematologist, correctly persevered and argued that in the long term it was better for the technicians to do the day-time

work when helpers were available than to be left at night with no help. In this way his Senior Technical Staff became increasingly proficient and they in turn taught their colleagues. After about two months of these difficulties the problems resolved and now technicians at Glasgow Western Infirmary are as good as our own staff in the recovery work. Since some cells were required two or three nights per week it was decided to recover cells at 5 p.m. from two units of blood. These are now set aside at 4°C and are used if required. If not, they are kept and used for matching to the serum of any suitable patient in the hospital who may require cells the following day. In this way the use of frozen cells has been given a shelf life of at least 24 hours. At weekends it was initially accepted that cells should be recovered every day but with the acceptance of a longer shelf life cells or 36 and even 48 hours post recovery storage at 4°C have been used without any adverse effects in the recipients. Reference has already been made to the measurements which would suggest that saline suspended red cells can be stored and used for up to 48 hours. With additives and metabolic support such as glucose supplements, adenine, plasma or albumin solutions some authors have suggested that recovered cells are viable from 7-14 days if kept at 4°C (Tullis et al 1960; see vol.1.).

The extension at the Western Infirmary has therefore proven of great service to patients and has been accepted by technical and medical staff without difficulty. Dr. J.D. Briggs, Consultant in Renal Diseases has been a main advocate of their use in order to minimise any hepatitis risk and reduce

the incidence of antibody formation to leucocyte and platelet antigens. The steady increase in work load is seen from the summary table of clinical cases. Renal cases are shown from 1973 separately for the Western Infirmary only. Glasgow Royal Infirmary and Stobhill renal cases are shown as a continuing and increasing commitment which formed part of a talk given to Haematologists in Belfast in February-March 1974. Since the introduction of frozen cells to dialysis patients in 1971 there has been a steady fall in the number of patients with antibodies to leucocytes. Some patients, because of previous pregnancy, or transfusions before referral to the renal unit and previous failed transplant continue to exist on the transplantation programme and these can be spared the problems of (a) further sensitisation and stimulation of antibodies to leucocytes and (b) adverse non-haemolytic reaction to blood transfusion. Dick (1975 personal communication) has reported that three years ago 25% of all patients on the transplant panel had leucocyte antibodies and that now about 10-15% of patients have such antibodies - mostly due to failed previous transplant, pregnancy or conventional transfusion. Briggs (1975 personal communication) reported to a discussion group at Glasgow Western Infirmary that he had analysed up to 1975 the failure rate of cadaver kidney transplants. His data (P1.6/1-4) excluding initial failures due to surgical difficulty and not due to immuno rejection showed that there were some 86 accumulated graft failures over the 3 years. Where there were two or more leucocyte antigen incompatibilities the graft rejection was greater than if there were less than two such incompatibilities (similar to data of Dausset (1974) and the three year experience of the

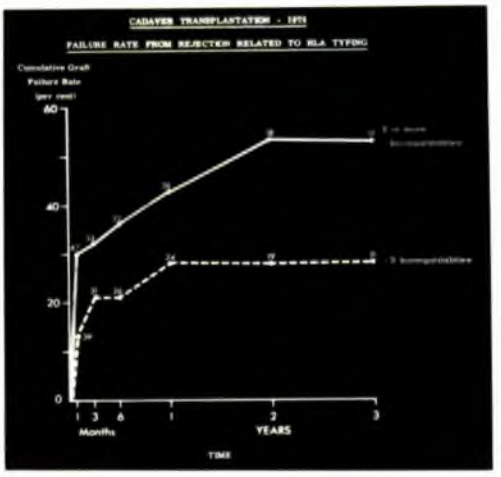


Plate 6/1

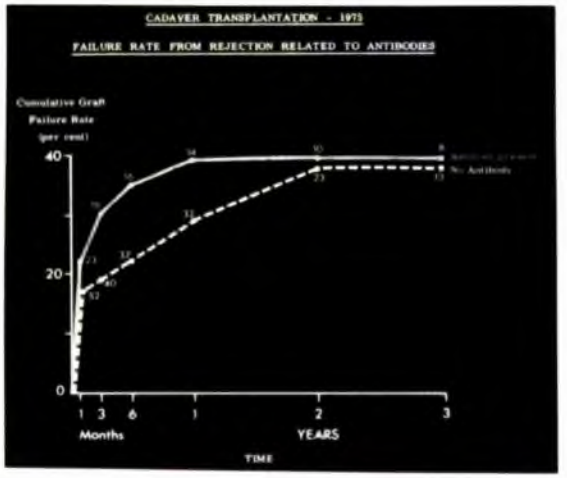


Plate 6/2

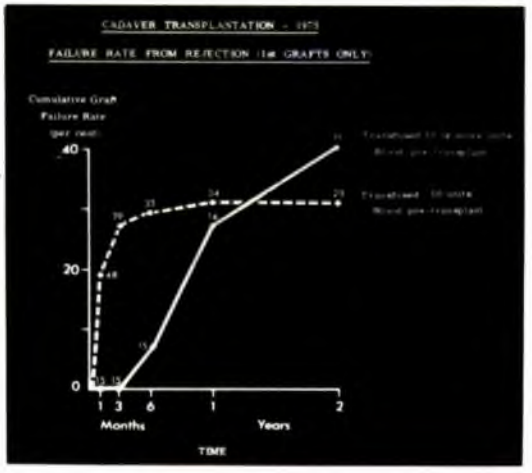


Plate 6/3

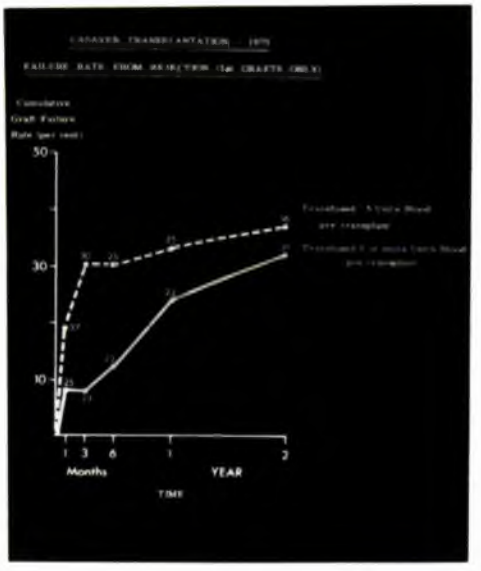


Plate 6/4

Plate 6/1 - 6/4. Glasgow Transplant Recipients Data

London Transplant Group, Oliver et al 1972). In the same data patients with pre-existing antibodies to leucocytes had a higher rejection rate in the first two years where they were then joined by a similar rejection of kidneys in patients without preformed antibodies (similar to Jeannet et al 1970). (Plate 6/14). In trying to assess the effect of blood transfusion he divided his data into those patients who had had more than 10 transfusions and those who had less than 10 transfusions of frozen cells (minimal transfusion). The data (Plate 6/15) show a better survival for the minimal transfusion group up to 1 year but thereafter they are overtaken by the excessive transfusion group who go on to a higher failure rate at 2 years. Further analysis of the data (Plate 6/16) show that patients given less than 5 units of bank blood or frozen cells had a more rapid early rejection within the first 6 months of transplant but that within the two year period the excess transfusion group has reached almost the same rejection rate.

These results, so far as actual transplants are concerned, would tend to support the recently argued case that patients given whole banked blood have a better early prognosis than those given minimal or frozen blood transfusion (see p Opelz and Terasaki 1974). It has been stated that the recipient should be allowed to develop antibodies in order to detect those "responders" who will pari passu make the MLC;B antibody. These will then be detected in the pregraft matching tests (if time permits) and the patient be spared a useless graft and needless operation which may jeopardise his chances when a more compatible kidney becomes available. It has to be remembered however that such patients are deteriorating all the while they are lying around with

their MLC.B antibody and that crucial time may be lost waiting for the ideal donor. MLC.B is said to develop in a small minority of patients (Senger et al 1974) and it may be these that are somehow preferentially prejudiced during the first few months of after a surgically successful graft. Thereafter the two groups of transfused and non transfused patients' failure rates approach one another and beyond two years the position is reversed.

These data are difficult to evaluate but I have attempted to try and reconcile them with recent observations in other centres which suggest that there is some graft rejection enhancement. These data certainly suggest this may be a possible explanation at least in the early stages of transplantation. Nevertheless one is still left with reports of hyperacute rejection associated with pre-existing leucocyte antibodies in the recipient (Kissmeyer-Nielsen 1966; Williams et al 1968; Pierce et al 1971; Terasaki et al 1971; Braun et al 1972) and the conflicting data concerning the influence of the histocompatibility identity or non-identity of the donor and recipient, (Murray et al 1974). Callender (1974) in a series of 185 renal allo-transplants found no statistically significant difference in allograft survival in one group with anti-leucocyte antibodies and a negative anti-donor cross-match when compared with a carefully matched group of recipients with no anti-HLA antibodies. Dausset (1974) in an analysis of 918 cadaver renal transplants noted that in those patients who had cytotoxic antibodies, pre-operative blood transfusion did worsen graft survival. Jeannet et al (1970) noted the early acute rejection in those with humoral antibody at the time of transplantation and those who developed antibody

in response to their graft had stormy clinical courses leading to rejection.

Miller and Caywood (1973) showed that alloimmunisation to leucocytes was minimal when frozen cells or leucocytes poor blood was used although most now accept that there is a minimal sensitising dose of leucocyte antigen. (Miller et al 1973; Lucas et al 1970; Perkins 1974).

Morris et al (1968) showed no relationship between the number of transfusions of bank blood and the early course of the graft as judged by rejection crises. In contrast to the previously quoted enhancement by blood transfusion they in fact believed that transfusions may cause immunologic unresponsiveness in renal allograft recipients due either to immunologic tolerance or enhancement. If so, they stated, tolerance being a state of specific unresponsiveness produced by treatment with antigens seems the most reasonable explanation. Similarly Myburgh (1974b) concluded that because there was an abrupt but transient anamnestic increase in graft-specific antibodies following transplantation associated with a very satisfactory clinical outcome, that an enhancement phenomenon may operate.

The poorer graft survival in multi-transfused patients was noted by Myburgh et al (1974a) in patients sensitised to more than 70% of a panel of random (screening) lymphocytes.

Opelz and Terasaki (1974) in stating that unresponsive (antibody producing) recipients of multiple blood transfusions have an unusually high rate of cadaver transplant survival, nevertheless also stated that pre-sensitisation of organ recipients prior to surgery has a deleterious effect on organ survival.

What is needed is a means of testing for those likely to

respond by making anti-HLA or MLC,B. The two would seem to be closely connected. Identifying these individuals by priming them with blood only diagnoses the inevitable rejection of the transplant. Failure to identify it may buy valuable time for the patient and could only be of value if it were shown that (a) many patients with MLC,B existed as a result of pre-transfusion and (b) many non-reactive donors in the MLC test were available. The evidence suggests otherwise. Indeed Senger et al (1974) concluded their summary on the relationship of blood transfusion to the appearance of mixed leucocyte blocking factor activity by saying that MLC,B "may play a minor role in the acceptance of renal allografts in man and means to induce blocking factor without deleterious lymphocytotoxins have to be explored before blocking factor can be used effectively for the clinical transplantation". The protagonists of using leucocyte containing blood have significantly I believe, not stated how they would ensure that recipients are always given histoincompatible MLC,B producing blood! Perhaps it is their intention to HLA and MLC type all donors of blood and organs for such patients. Assuming each renal patient requires on average 4 units of blood per annum (Briggs personal communication) they have a formidable task ahead to improve the low response rate described by Senger et al (1974).

What is needed is less transfusion not more or if their is to be more, blood which is free of leucocytes, platelets, plasma proteins, alloagglutinins, microaggregates, unwanted cations and is probably free of detectable hepatitis virus and according to most workers, is physiologically superior in several respects. (Ogden 1972, Polesky, 1972.)

None of our patients have experienced any adverse effects during the infusion of recovered cells in the way of any rigors, pyrexia or urticaria. This is in agreement with most other blood freezer experience. Tullis et al (1960) noted that in a series of over 1500 units transfused the incidence of reaction was less than 0.3% compared to an average of 5% for conventionally stored ACD blood - a fifteen fold decrease. Similar results were recorded by Tullis (1958) in patients who had had previous reactions and who suffered from fatal haematological and malignant diseases where such reactions were not unknown and commoner than usual. This attribute of the frozen blood is, of course, not a special property of frozen cells but represents a useful by-product i.e. the allergens are incidentally removed in the processing.

It has already been stated that the scourge of the renal dialysis patient, his attendants and fellow patients is the hepatitis virus B and any measures aimed at reducing this including non-transfusion or frozen blood transfusion can only be beneficial. The reports of unpleasant and sometimes severe reactions to histoincompatible blood in patients with preformed antibodies to leucocytes makes every haematologist pay attention and seek to minimize this risk and not to enhance it (Brittingham and Chaplin 1957). (Perkins et al 1966).

Finally I can do no better than quote from a recent paper by Fabre and Batchelor (1975) who in discussing the work of Opelz and Terasaki say "whatever the outcome of this controversy in the short term it is likely that preventing the immunological sequelae of blood transfusion will become increasingly more important as techniques of immunosuppression improve".

STATEMENT ON PRETRANSPLANT TRANSFUSION

I read with interest the Leader in Lancet (15th November 1975) and the letter by Professor Shaldon (20th December 1975) advocated the severe restriction of blood and especially frozen blood in patients awaiting renal transplant. The Rosenheim report (1971) established the principle that minimum transfusion was desirable and most authors would accept this because of the peculiar circumstances surrounding hepatitis outbreaks in dialysis units. There is however a requirement for patients with leucocyte antibodies due to previous pregnancy, graft rejection and transfusion of blood. Many patients are not in need of transplant when first seen and suffer from treatable haematological disorders. Vivid descriptions exist in the literature and in our experience of the relief from non haemolytic transfusion reactions in such patients given leucocyte depleted blood. Opelz, Mickey and Terasaki (1973) found a direct relationship between the quantity of blood transfused and the development of antibodies to HLA. I heartly agree with Professor Shaldon in his analysis of the doubtful comparison of a carefully studied control population in one centre and a less carefully studied transfused group obtained from a larger number of different centres in a variety of places (90 patients entered by 22 different centres). It is recognised that in addition to causing febrile reactions (Chaplin et al 1959) the antibodies to the HLA system can jeopardise the survival of organ transplants (p 38 , Kissmeyer-Neilson et al 1966; Pierce et al 1971; Terasaki et al 1971; Myburgh et al 1974) and compromise the response to component transfusions (Yankee et al 1969). Routine testing to detect and prevent

HLA sensitisation is time consuming and hardly feasible for every case. As a working alternative numerous procedures to prepare HLA poor blood have been developed (Mitchell 1976). The response to the HLA antigens is dose related (Perkins et al 1966; Brittingham and Chaplin 1957) and these may even be present in the plasma despite white cell removal (Van Rood et al 1970; Helgeson et al 1972; Crawley et al 1973).

Patel and Terasaki (1969) associated hyperacute renal transplant rejection with leucocyte antibodies and Perkins et al (1973) have suggested that as many as 40% of patients awaiting renal transplantation may be already immunised primarily because of repeated transfusions of concentrated red cells which do not as is popularly believed, free the transfusate of leucocytes (Goldfinger et al 1973; Tenczar 1973). No one yet knows the exact minimum dose of immunogen required to stimulate a response to foreign HLA material (Opelz et al 1973). It is variable and based on numerous differences in donor HLA type, recipient HLA type and other treatments, past and present.

Freezing and thawing blood does not remove all of the HLA material although it is the single most efficient means of depletion yet devised (Lucas 1970; Crawley and Valeri 1974; Millar and Caywood 1973; Perkins 1974; Perkins et al 1973). Schechter et al (1972) demonstrated lymphocyte responses in patients receiving allogeneic whole blood which did not occur in patients receiving frozen cells. This suggests that even the small amount of HLA material remaining was altered in its immunogenicity as well as being quantitatively minimised. Defrosted lymphocytes were shown to exclude trypan blue, gave no phytoagglutinin response, no mixed lymphocyte reaction nor uptake of tritiated thymidine.

Despite the claim by Opelz (1974) that whole blood may produce an antibody response which enhances graft rejection most other authors consider that the evidence is in favour of the formation of humoral antibody in association with hyperacute graft rejection (Jeanette et al 1970; Pierce et al 1971; Miller et al 1973; Tenczar 1973; Dausset 1974). A monthly study by Suarez-Ch and Jonasson (1972) showed that 32% of renal dialysis patients (men 23%, women 45%) had preformed leucocyte antibodies mainly due to whole blood and previous pregnancy. Nevertheless even leucocyte poor blood over a period of two years eventually resulted in 7 out of 135 patients developing leucocyte antibodies.

Morris, Ting and Stocker (1968) in 43 patients found no relationship between the number of transfusions and the early course of renal graft rejections. They considered that blood transfusion may cause immunological unresponsiveness due either to immunologic tolerance or enhancement. Sengar, Rashid and Harris (1974) showed the presence of a mixed lymphocyte blocking factor (MLC;B) in the sera of pregnant women, previously transfused patients and in chronic uraemia and recipients of renal allografts. The development of MLC;B seemed to them to be related to the formation of lymphocytotoxins. They considered it played a <u>minor</u> role in the acceptance of renal allografts in man and suggested that identification of lymphocytotoxin producers (responders) would reveal patients likely to produce MLC;B and have less likelihood of rejecting and graft since this would be revealed by the in vitro mixed lymphocyte test and an already doubtfully acceptable kidney transplanted. As a corollary, if this is of "minor" importance the "major" important finding is that one should not try and identify such responders since the majority of patients who

have not developed preformed antibody should do well. Callender (1974) in 185 renal allotransplants noted 32 recipients who had HLA antibodies and a negative anti-donor cross-match. Compared to graft recipients, who did not have any HLA antibodies, revealed no difference in the allograft survival. Myburgh (1974) found that presensitisation to HLA resulted in lower graft survivals. They further found that the lymphocyte response in patients to donors may be negative despite unequivocal serological evidence of pre-existing graft specific sensitisation and a positive lymphocyte response could be associated with a satisfactory clinical outcome.

Opelz, Mickey and Terasaki (1973) themselves stated that 90% of the patients in 11 haemodialysis centres in Los Angeles were dialysis patients, 78 had developed antibodies to HLA due to previous pregnancy, transfusions or transplants. Leucocyte poor blood greatly improves these statistics. Such patients waiting for transplant may experience a slow or accelerated decline in renal function. The availability of a suitable graft may take some time. Assuming there is sufficient warning and the time consuming MLC testing can be done (the ideal situation) the alleged benefits are likely to be minor. In attempting to realise these benefits valuable time may be lost and, equally, blood transfusions to help maintain the patients condition so as not to jeopardise his chances of survival until a more compatible kidney becomes available. Opelz and Terasaki (1974) in declaring that unresponsive recipients of multiple blood transfusions have a high rate of cadaver renal transplant survival, nevertheless also state that pre-sensitisation of organ recipients prior to surgery has a deleterious effect on organ survival. My case

therefore is that there is every reason to minimise the transfusion of patients awaiting transplant but that where it is necessary, leucocyte poor and preferably defrosted blood should be considered as the most suitable. If present trends in transplantation continue (Weiden et al 1975) then the physiological superiority of frozen blood is assured for the reasons that have been outlined. The protagonists of using leucocyte containing blood have, significantly I believe, not stated how they would ensure that recipients of blood are <u>always</u> given <u>histocompatible</u> MLC;B producing blood. If HLA and MLC typing of all donors of blood and organs is to be done then if each renal dialysis patient requires, say, 4 units of blood per annum there is a Herculean task ahead to achieve the low response rate and detect the responders and non responders. The advent of frozen blood banking by practical means removes many of the hitherto held objections of icterogenicity, immunogenicity to leucocytes, platelets and plasma and ensures cation removal, excellent oxygen transport function, normal survival and retention of optimum function. The quality of life for the patient is just as important pre-transplant as in the post transplant period. The longer the survival the greater will be the requirement for long term support. One must also remember the patient who may experience a failure of renal graft due to non-immunologic causes (Dossetor et al 1967). More recently Mittal et al (1975) have written of factors other than HLA which may play a significant role in the outcome of allografts and postulate that other genetically determined system may be relevant.

PART VII

THE LOGISTICS OF FROZEN CELL BANKING

"Through this distemperature we see
The seasons alter: hoary headed
frosts
Fall in the fresh lap of the
crimson rose".

Shakespeare, A Midsummer Night's Dream.

In this section I propose to deal with certain developments and techniques introduced into frozen cell banking as a result of our experiences and to show how these have related to the work of others and I believe materially improved the system.

Recovery and Inventory Control

One of my first papers in frozen blood concerned the method of storage of frozen cells and how they could be easily identified at short notice. The paper enclosed with thesis shows:

(a) A system for dividing the storage area of the main dewars (CPV 250) into a series of compartments each with its own identity numbering system.(Plates 7/1&7/2.)

(b) Sufficient accommodation for two rows of cans, one on top of the other, one sitting in the liquid phase approximately 2 inches above the base plate and the other sitting on top of this in the vapour phase but being influenced by the lower one by simple physical conduction.(Plates 2/9&7/3.)

(c) A system of freezing small sub-samples for matching and other tests using the residue of glycerolised blood to fill pilot tubes which are snap frozen in liquid nitrogen and separately located in specially designed compartments and filing racks. Retention of antigens up to 8 months was reported (Mitchell 1971) but has since been extended for up to 4 years without loss.(1 7/4.)

(d) A simple but effective flotation collar for ensuring adequate rapid thawing by keeping the cans upright and bobbing about in a water bath.(Plate 7/5.)

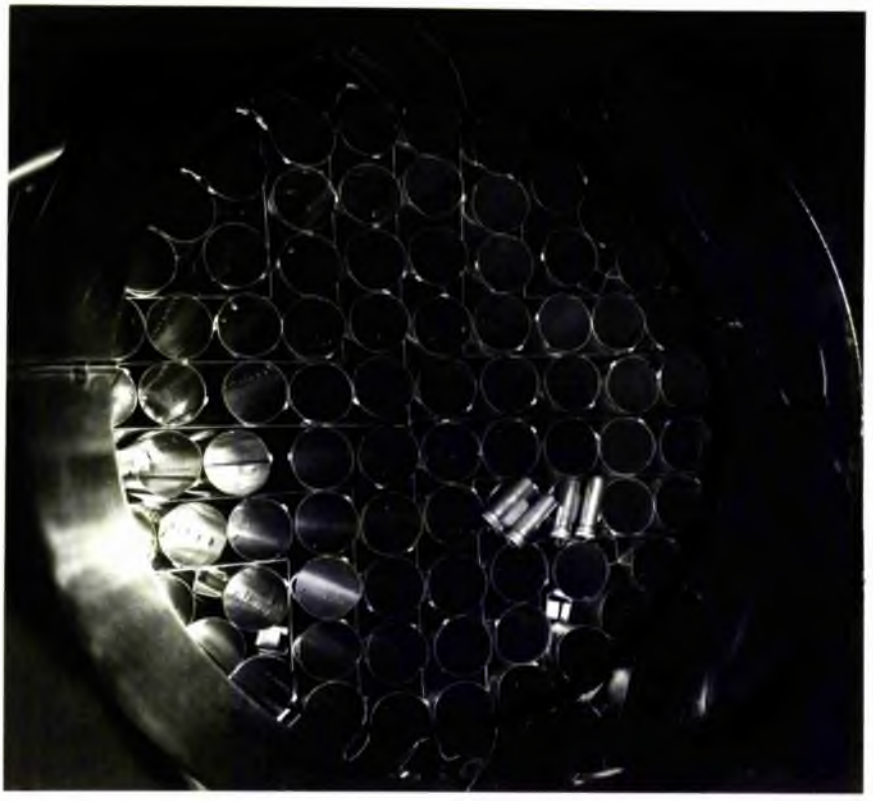


Plate 7/1

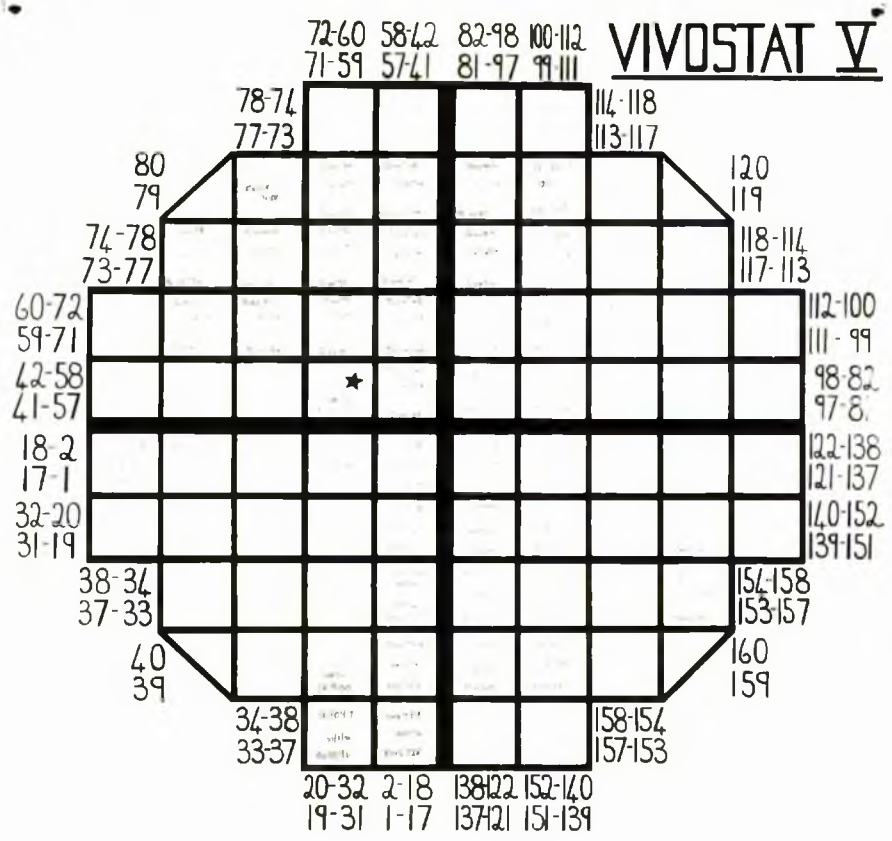


Plate 7/2

Plates 7/1 and 7/2. Storage System for Large Vessels

Plate 7/3. Main Cryogenic Blood Bank Store

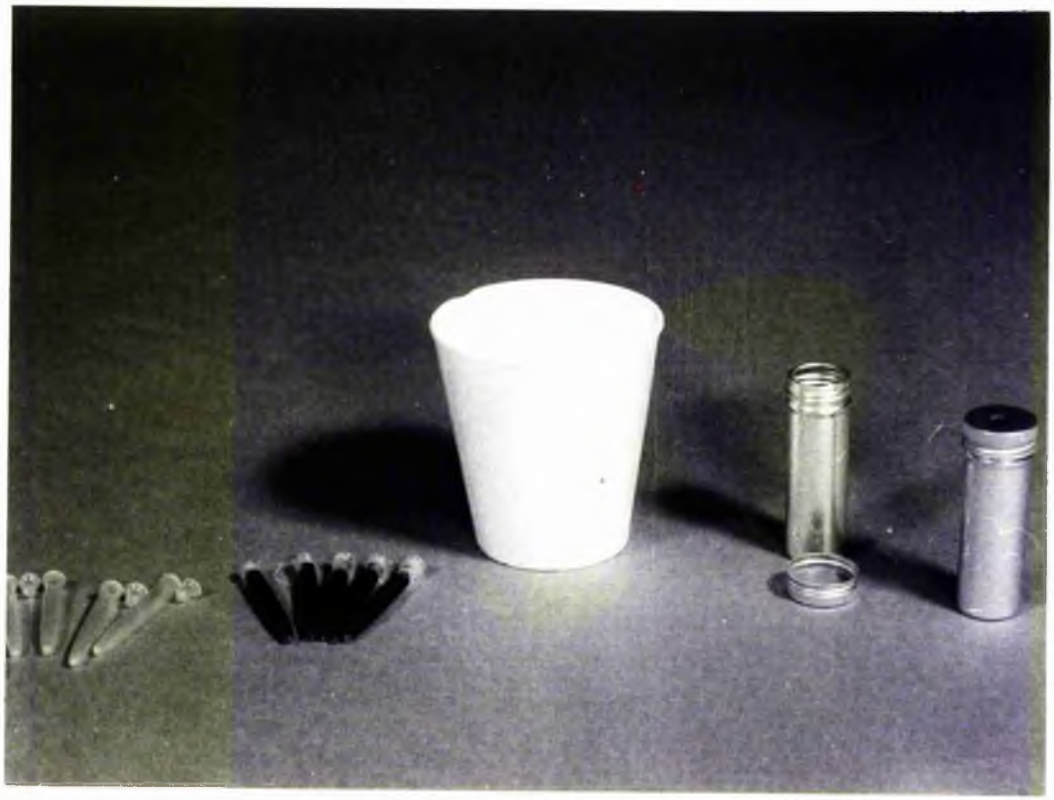
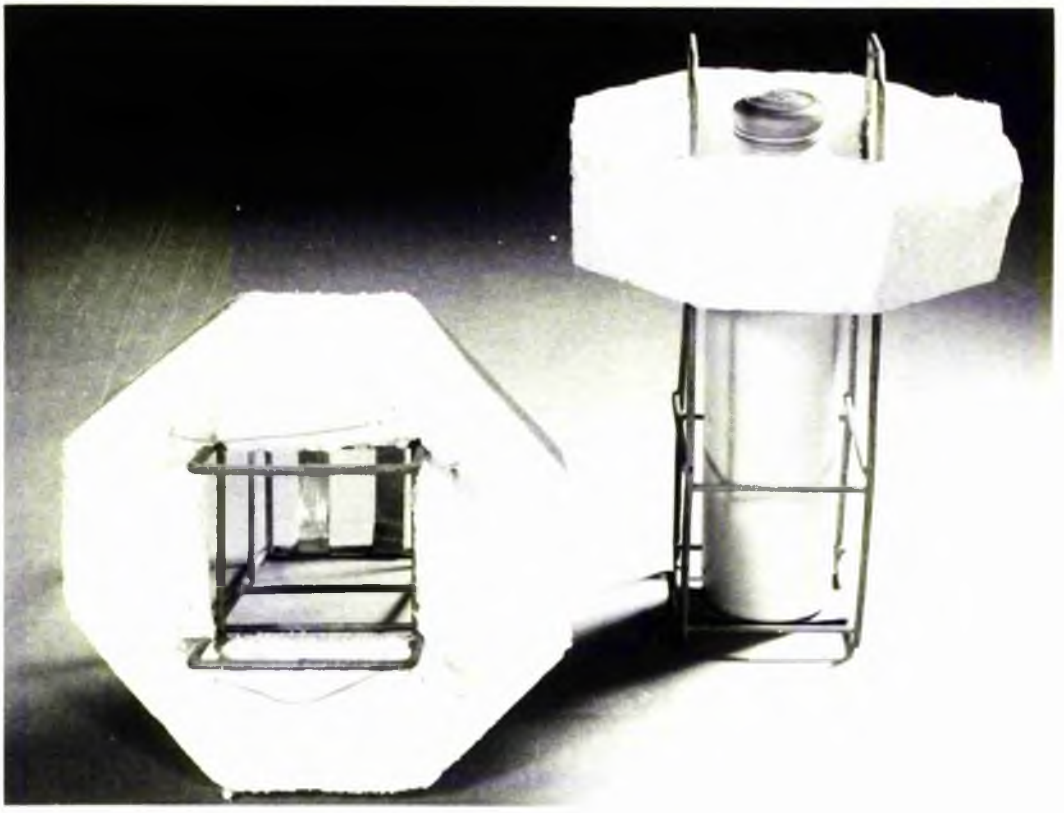


Plate 7/4. Freezing Sub-samples.



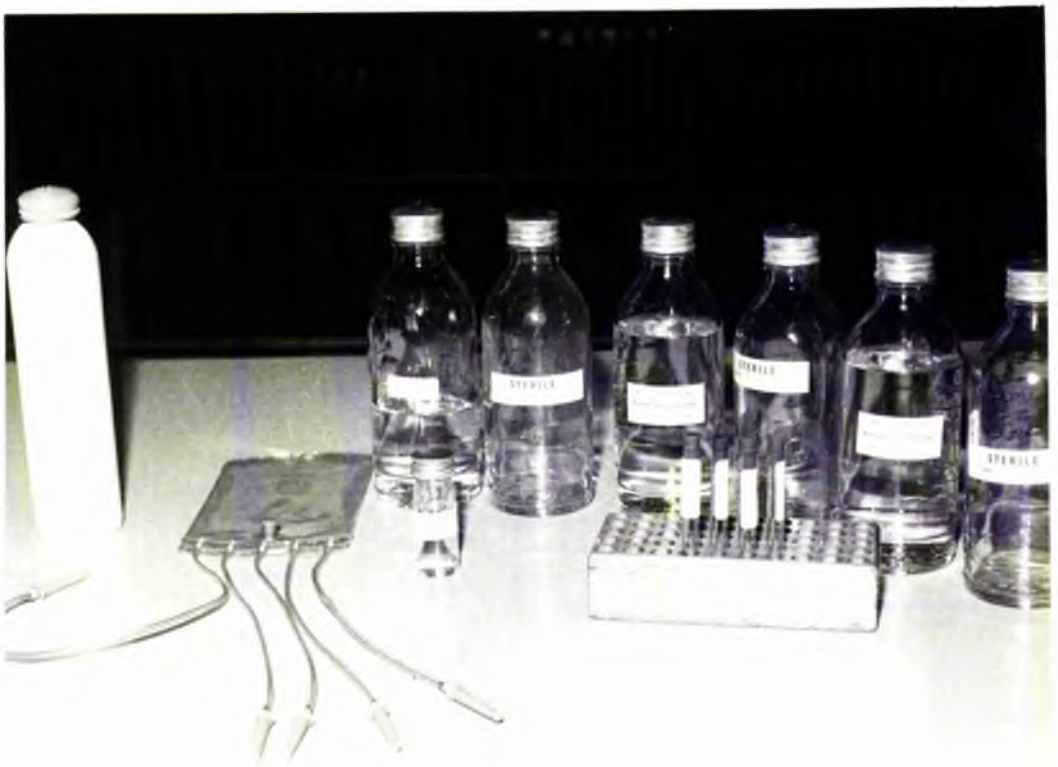


Plate 7/5. Floatation Collar and Recovery Fluids

A A_1 A_2 B O AB A_1B A_2B C C^w D D^u E c c^v d e M N S s P_1 K Lu^a Le^a Le^b Fy^a Fy^b Wr^a Jk^a k Kp^a Kp^b Cl^a

Donor's Name ..... Donor No. ..... Group ..... H.A.A. ..... L.N. No. .....

Address ..... D.O.W. ..... Container ..... Glycerol Batch No. .....

..... D.O.F. ..... Sorbitol Batch No. ..... Saline Batch No. .....

Donation	Date	Total Vol.	Vol. P.C.	Vol. Supernate	Gm Hb Supernate	Gm Hb Total	P.C.V.	W.B.C.	Film	Remarks
Original										
Pre-Freeze after Glycerol										
Post Thaw										
W1. Sorbitol										
W2. NaCl										
W3. NaCl										
Product										

BACTERIOLOGY

Agar		Robertson's R.T.	Brewer's 35°C	Robertson's 37°C
R.T.	37°C	Blood Agar	Blood Agar	Blood Agar
		Room Temp.	Anaerobic	37°C

Plate. 7/6. Donor Record Card.

GLASGOW AND WEST OF SCOTLAND BLOOD TRANSFUSION SERVICE

Patient's Name _____

Address _____

_____

Date of Birth _____

Hospital _____

Date of Transfusion _____

Platelet Antibodies _____
(Complement Fixation)

Leucocyte Antibodies _____
(Microlymphocytotoxicity)

Product Film _____

Bacteriology _____

Donation No.	Date of With-drawal	RED CELL PROFILE																				Final Hb	Final Vol	Total	HAA
		ABO	Rh	M	N	S	s	P_1	K	Lu	Le^a	Le^b	Fy^a	Wr^a	Jk^a	k	Kp^a	Kp^b	Cl^a			g/100ml	PC	Hb/%	
Patient																									

Signed _____ Date _____

Plate 7/6a Recipient Record Card.

(e) A punch card record keeping system for all donors and recipients.(Plate 7/6.)

All of these innovations have since been modified and will be described in detail.

Storage System

As the size of frozen cell bank increased the original two vivostats were insufficient to store the number of cells. Aluminium tubes of the correct gauge and length are not easy to obtain and costs are high. We had received a Christmas Calendar in a cardboard tube and this gave us the idea of using cardboard tubes instead of aluminium tubes which incidentally were modified by Union Carbide Co., U.K., who fabricated them in mild steel and started selling these as part of their liquid nitrogen freezing 'package' system. The cardboard tube manufacturer had inscribed his address inside the tube and we were able to obtain unlimited supplies of plastic coated cardboard tubes of the correct diameter which could be cut to length. These are a fraction of the cost of aluminium and have served the purpose well with no special problems. A notch is cut out of the bottom of the tube to allow free circulation of nitrogen and a piece of 22 gauge wire is stretched across and one inch above the notch. In this way cans are held securely upright in the same basic configuration as shown in the reprint of the original method.(Mitchell et al,1972.)

Furthermore when the system became known to other users they were quick to adopt it and again a commercial company put out such tubes as part of their 'package deal' for frozen cells. When we were ready to introduce the extension of frozen cells to Glasgow Western Infirmary, Haematology Department, the author with the Regional

Transfusion Director, Dr. J. Wallace, met with the Haematologist, Dr. H. Hutchison, to discuss the location and transport of the blood and its storage at the Western Infirmary. Part of this was to use the smaller vivostat L.R. 40 because of problems of space and the amount of manhandling of vessels due to the haematology working area being only approachable by negotiating a series of split levels and stairways through a busy outpatient area. The L.R. 40 vessel with inner porous spacers (blocks of special absorbent) can be charged with liquid nitrogen yet not in any danger of spillage of free liquid nitrogen. With the spacers in position it is still possible to contain 20 units of frozen cells in the upright position and the cardboard tubes again can be arranged to fill the vessel at the same time mutually supporting one another with no danger of falling over. Numbering is easy and consecutive starting clockwise from a reference starting point. A plan of the inner vessel is sent with each load of frozen cells so that location of any unit is easy Plate 7/7&7/8. This numbering and location system is similar to that outlined in the reprint for the larger CPV 250 vessel.

When the frozen cell storage system was introduced at Inverness and Aberdeen, we were able to send to each centre a complete inventory entirely copying this method. The same method has been adopted by Guy's Hospital Frozen Cell Bank and the Leeds and Edinburgh Blood Transfusion Centres. This has meant two advantages, the first being a uniform system of inventory keeping and the second considerable saving of time and money trying to locate aluminium tube manufacturers. Originally we were worried that the cardboard tubes might

Plate 7/7. Storage in Small Vessels.

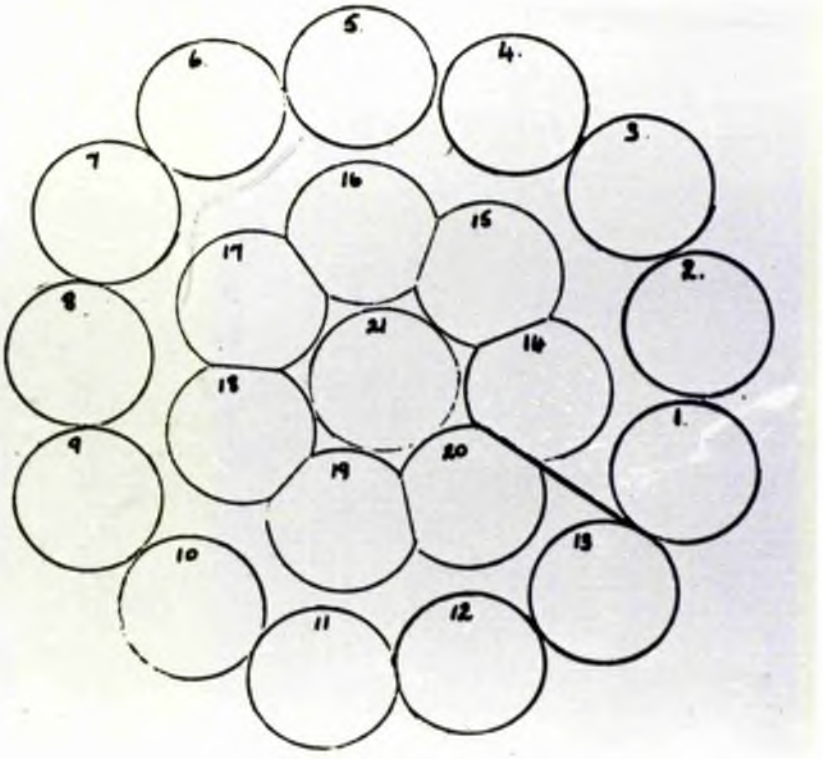


Plate 7/8. Inventory Plan for Small Vessels

not be strong enough but in liquid nitrogen they freeze solid and form a rigid support. The occasional one which becomes fractured or frayed can be easily replaced.

To compliment this mechanical stock holding system a card index using punch cards was used for noting donation information with a separate similar card for "recovery from bank" information (see enclosed samples). The donor cards can be used for a manual sort out of any particular blood group combination using a long wire to 'sift' the appropriate cards of donations containing any particular antigen or alternatively to avoid selecting any particular antigen to which a potential recipient may have an antibody.

This card system is similar to that used by the American Association of Blood Banks for their rare antigen donor panel but was introduced before this was known. In fact, I recalled seeing a similar system in the Cytology Diagnostic Laboratory of Dr. H.E. Hughes at Glasgow Royal Infirmary many years ago. Because of the rapid increase in the use of frozen cells in the United States of America, inventory systems have now been computerised (Rowe et al 1970, 1971: Roberts 1975). This is obviously very costly and beyond our resources. At the moment any donation put into nitrogen is noted in the West of Scotland's Computerised Donor File as having been issued to RUM (author's initials!). Thereafter the computer will not be unduly worried if the donation is not returned unused at 28 days after withdrawal or is seemingly given to a patient when it is outdated on the day of issue! If the donor returns to give a later donation the computer programme will signal that the donation previously went to RUM. This is valuable since it means that screening and antigen testing antisera need not be

constantly drained away in testing random donors and the RUM stocks become enriched with multiple donations from the same donor (see Table 6/1 on donor numbers and distribution of donation types).

Gradually in this manner it has become possible to obtain the types of blood categorised by Rowe et al (1971; Grove-Rasmussen and Huggins 1973).

1. Rare blood which is needed for patients of a specific blood type whose incidence occurs in 1 in 250 or more donors, e.g. cellano negative, I negative, Kp^b negative donations.
2. Freshly typed blood which is typed for 20 or more different red cell antigens for patients whose sera contains multiple complex antibodies or for boosting human volunteers (see anti-D volunteers vol,1). Once a patient's antibodies have been identified suitable units can be chosen manually using the card sort out described above. Eventually it will be possible to do this by computer. Because of shortage of valuable antisera it is not always possible to fully type all donors. When bone marrow donors and platelet/leucocyte donors are histocompatibility antigen tested it will be possible to add this information to the donor cards and records.
3. Partially typed blood is usually only typed for group O and rhesus phenotype but recently we have considered extension of this to include ABO group and the Lewis system to improve the number of potential donors for the frozen cell bank and to cope with the apparent increase in the number of ante-natal patients from the Asian Sub-continent who have a high incidence of Le (a-b-)

phenotypes associated with anti-Lewis antibodies (Race and Sanger 1975). Many of these cases dealt with recently have been admitted to hospital at short notice and put additional strain on our resources. It is hoped that as sufficient donations are placed in the frozen bank that this will present less of a problem. Partially typed blood is usually issued for renal dialysis patients and those where there is no red cell antibody problem. Blood in this category is obtained on a regular basis from routine donor sessions, previous computer records that previous units were frozen, donors with antibodies where the red cells only are stored with removal of residual antibody during processing and random donors at times of excessive intake such as Student Charities Campaign Collections. At the time of writing, 24% of the blood in our frozen cell bank is fully profiled as in category 2 above and the following rare donations have been stored, KK, 22, R2R2, 3O, Kp^a, Kp^a 2, Oli 1, autologous units 3.

Demarjian and Kliman (1972) in outlining their freezing blood programme for the State of Massachusetts commented that since blood was given by volunteers there were inevitably peaks and valleys of supply corresponding with annual holidays, epidemic illness, industrial disputes, bad weather conditions etc. Although most authors have given this as a reason for introducing frozen cell banking this has not been a major problem in our Region (Fig.7/1, Haynes et al 1960). Nevertheless Huggins (1974) at the 11th Annual Meeting of the Society for Cryobiology Meeting in London commented that one third of his annual usage was frozen cells and that this had reduced the outdating of blood in Massachusetts General

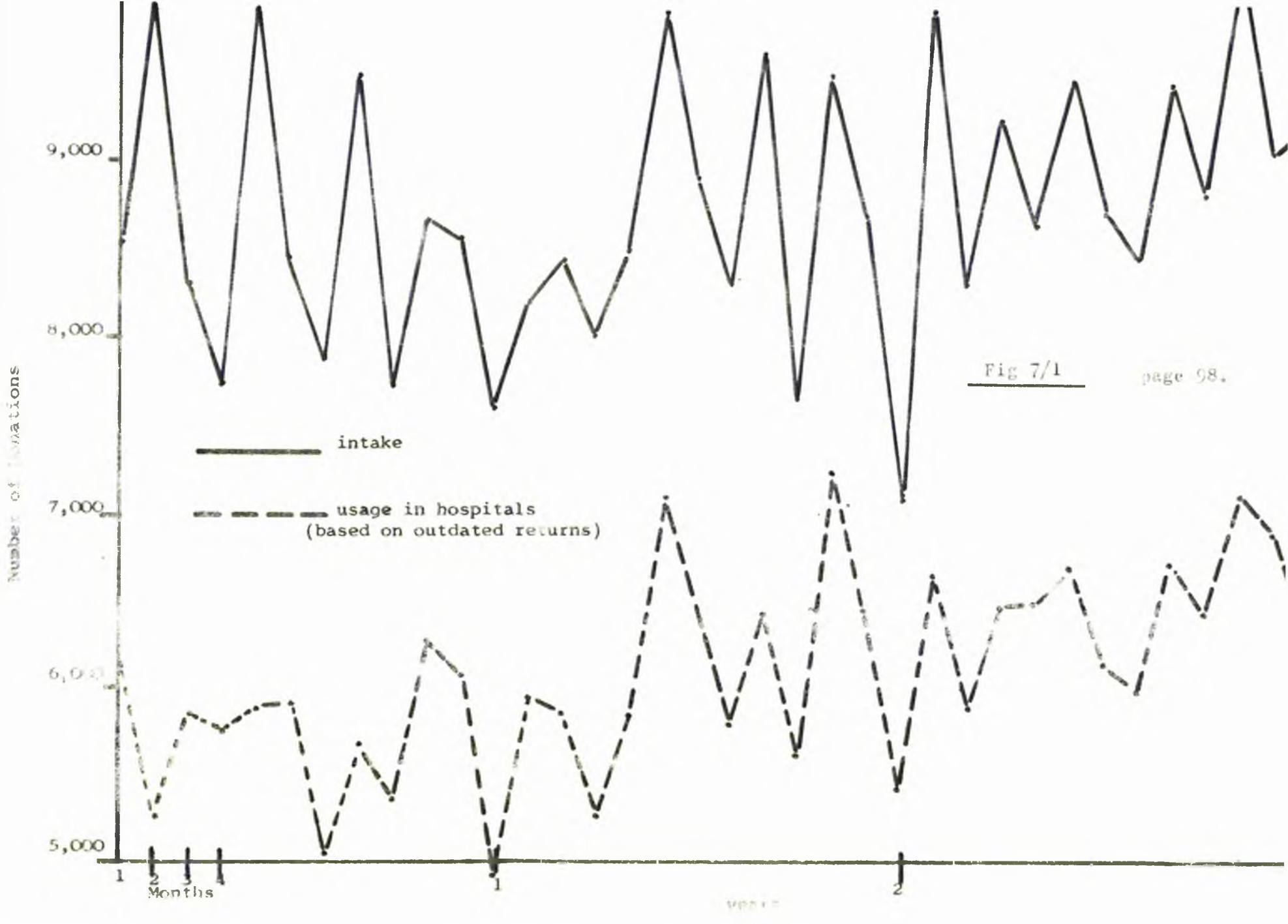

9,000
8,000
7,000
6,000
5,000
Number of Donations
intake
usage in hospitals
(based on outdated returns)
1
2
3
4
Months
1
2

Fig 7/1

Hospital from 9% to less than 1%. At the same meeting Rowe spoke of critical shortages of blood in the State of Massachusetts in 1973 which had only narrowly prevented imminent breakdown of the supply. Meryman also spoke of the introduction of the low glycerol rapid freeze technique to 32 centres in USA by the American Red Cross. Demerjian and Kliman (1972) recorded that despite the complexity of equipment required, the preparation of solutions, high degree of quality control and costs of 5-10 times that for conventional banked blood, 12,000 units had been recovered from frozen banks for patients in The Blood Research Institute (Dr. J.L. Tullis), the U.S. Naval Hospital Blood Research Laboratory (Dr. C.R. Valeri) and the Massachusetts General Hospital (Dr. C. Huggins). Decentralization of supply was done by setting up 3 sub-regional centres so as to cover as wide an area as possible and yet still keep the basic equipment, technical staff and quality control under their direction. This latter is very similar to the thinking which prompted us at this Regional Centre to decentralize some of the recovery work to the two main Glasgow Teaching Hospitals. One of these is already fully operational and the other is about to start thus bringing frozen cells nearer to the point of delivery to the patient.

Transport of Frozen Blood

Since these extensions of the frozen cell banks mean that blood has to be transported over a distance and remain in the frozen state, I was anxious to know if L.R. 40 vessels filled in the manner described above would maintain the temperature at below -150°C and if so for how long. In mechanical refrigeration as well as the problem of

electrical failure, Tullis et al (1958) described a temperature difference between the top one third of the storage cabinet which was about 20% warmer than the bottom with a wide swinging fluctuation over a 30 degree range in cabinets operating at -120°C. These fluctuations could result in lost stock with lower yields of cells and higher supernatant haemoglobins. A thermocouple was inserted into a can of blood and the whole can placed in the frozen state into the L.R. 40 and kept in an upright position. The lid of the L.R. 40 was deliberately removed for varying lengths of time for 10 to 30 minutes and recording of the temperature was made on a slow moving chart paper in the Leeds and North Speedomax XL chart recorder. Temperatures remained stable over a period of 3 days. The lid was removed twice each day and for the 10 minute test the temperature did not vary although in the 30 minute tests the temperature rose to about -120°C. These findings clearly demonstrate that the system of storage is more than adequate for operational conditions of blood being kept frozen for prolonged periods (up to 3 days in our experiment) without having to top up the liquid nitrogen level and allowing technicians to take the lid off to extract a particular donation. It is inconceivable that anyone would need more than a few minutes at the very most for this which is the reason that the extremes of 10 minutes and 30 minutes 'lid-off' conditions were chosen. At the end of the experiment the unit was taken, thawed and recovered without difficulty. Apart from the small trial done between us and Brentwood R.T.C. referred to on p. only the British Army have done any similar work and blood has been sent by them to South East Asia by air (Valeri 1974).

Their results are not published but it is known that the L.R. 40 vessel has been used for flying cans of frozen cells across the Atlantic Ocean in pressurised military aircraft and that a similar system operates in sending blood to Germany. The North Atlantic Treaty Organisation (NATO) are currently stockpiling red cells in low glycerol liquid nitrogen banks. There are some reports of transport of frozen cells by the high glycerol -80°C method. In 1966 the U.S. Navy established frozen cell facilities in Vietnam, on shore at Danang and at sea aboard the hospital ships USS Repose and USS Sanctuary in order to evaluate the practicality of having a supply of frozen blood in a combat area as a supplement to liquid blood preserved in ACD (Moss et al 1968; Valeri et al 1966; Valeri 1969; Valeri 1974). Blood was transported to Vietnam via the Philippines packed in polystyrene containers with dry ice (carbon dioxide). Forty-three critically wounded service men received 307 units of frozen blood (+ 347 ACD) with no significant adverse effect on patient well being including plasma haemoglobin, bilirubin, platelet count, urine haemoglobin or serum creatinine. No such evaluation has ever been done with liquid nitrogen preserved cells although there would seem to be no reason to expect anything different. Valeri (1969) recorded that 1000 units recovered from a liquid nitrogen bank at -150°C had been prepared by batch washing and transfused without difficulty and later over 3000 units of frozen cells had been given to severely wounded servicement which proved clinically acceptable and technically feasible in a combat zone (Valeri 1969).Certainly since the introduction of the frozen cell bank at Glasgow Western Infirmary all of the 1134

units recovered by that department for 170 patients (see Fig.) have all been transported by road from the Regional Transfusion Centre in the frozen condition. Similarly no problems have been encountered in transporting frozen blood to Glasgow Royal Infirmary. Huggins (1970) in reviewing his method commented on the ease of transport at -80°C on an International scale.

There are reports of liquid blood being examined after prolonged journeys at 4°C in insulated boxes. Orlina, Button and Taylor (1958) transported such blood over thousands of miles by air and land, bus and train, with no deleterious effect. Krijnen et al (1970) described the collection of 5 donations of blood at 4°C in Finland being flown to Amsterdam where they were frozen in the frozen cell bank, recovered after 4 weeks and returned to Helsinki by air at 4°C. Radio chromium survival studies showed 85-93% recovery at 24 hours post transfusion and in vitro recovery was 91-95%. Krijnen and his colleagues had a special reason for wishing such information since they have established a central bank of frozen cells in Amsterdam to which donations of rare cells can be referred for long term storage (see Appendix). A similar scheme has been introduced by Jenkins and Blagdon at Brentwood which works well. Perrault et al (1967) collected blood in Ottowa, flew it by air 2000 miles to Halifax and back where it was frozen by Huggins' high glycerol -80°C method. Three days later it was thawed, reconstituted in saline, flown back to Halifax and return air freighted to Ottowa where it was transfused with normal survival ($T_{1/2}$ = 32 days).

Sullivan and Hammack (1965) reported the favourable use of frozen cells recovered in Boston and sent to their

hospital in Birmingham, Alabama. Grove-Kasmussen and Huggins (1973) thought that since the majority of centres using frozen blood have the same equipment it is possible to move frozen cells from one centre to another where it can be processed if needed and kept in storage (-80°C) until needed (see p. 100. This is a strange statement from a country where there are at least three quite different methods practiced in different centres and sometimes in the same centre. Nevertheless the sentiment is correct.

Tullis et al (1971) described the shipment of two units of frozen blood (-80°C) by air a distance of 2000 miles to demonstrate red cell stability whilst in the glycerolised state. In 1963 Valeri et al described the transfer of blood frozen at -80°C a distance of 3000 miles by commercial airliner in a 15 hour flight from Chelsea Hospital, Boston, Massachusetts to Oakland, California where they were thawed, processed and retained at 4°C. Ganshirt and Seidl (1974) in assessing the use of oxypolygelatin containing metabolically active resuspension medium stated that in their view frozen recovered cells could be transported anywhere in the World within 48 hours without detriment.

In our own studies we have recovered frozen cells in the Regional Transfusion Centre and transported them at 4°C by train and airplane to Inverness for boosting and immunising rhesus negative male volunteers to produce anti-D for immunoglobulin production. As well as transporting all around the West of Scotland, recovered cells have been sent by road using our own delivery vans to Dumfries and Dundee. On all occasions cells have survived in good condition and

been transfused without difficulty.

Modifications to the Storage System

Since it is good practice to always keep the pilot samples with their donations it became increasingly important to do this as the bank grew in size and it became more and more inconvenient to retrieve filed matching samples. The sub-samples are now stored in small tubes as described in the paper (**Appendix .**) but instead of filing them separately it is possible to attach the small labelled aluminium box containing up to 5 small labelled tubes, to the neck of the main donation can. The box has two holes in the lid and is labelled with the donation number. Thin wire through the smaller hole can be secured and serves conveniently for pulling the whole donation out of the vivostat (**Pl.7/9.**). The larger hole in the lid serves for equilibrating pressure and prevents any tendency for liquid nitrogen inside the container from causing explosive rupture in the event of the screw threads becoming blocked and stuck by ice formation.

This system allows the matching samples (up to 5) to be stored with the main donation. These can be used for matching tests in advance of thawing out the main donation and others can be used for other examinations such as storage properties of red cell antigens.

With the growth of the bank we tried to develop an automatic system for topping up the vivostats with liquid nitrogen. With the help of British Oxygen Company high and low level sensors were fitted to each of four vivostats and arranged to give an audible alarm in the event of exceeding the preset limits (2" and 7½" above the base plate) and to cause solenoid valves to open and close thus allowing liquid nitrogen from a pressurised (5 p.s.i.) main

Plate 7/9. Modified Storage System in Operation

500 litre storage tank (TWN 500) to recharge the vivostats. Because of the small diameter of the delivery pipes and the lack of adequate insulation along the flow path, every time the system operated much liquid nitrogen was converted to nitrogen gas which continued flowing until the pathway was sufficiently cold to permit liquid nitrogen to flow. Pressure build up at these times and formation of water ice further diminished the flow so that instead of delivering a calculated liquid fill at about 10 litres per minute only a trickle appeared and filling took an inordinate time (30 minutes for top up of only a few litres) with a technician standing by all the while. This system was further complicated in that gaseous losses of nitrogen were much higher and liquid nitrogen deliveries all the more frequent. The system was quickly abandoned as being unworkable in favour of a return to simple pumping at atmospheric pressure with the standard CSMPB pump (BOC) which delivers 6 litres/minute. Level alarms were also abandoned in favour of a quick dip with a graduated stick which on removal from liquid nitrogen shows a clear "frost line" indicating the level above the base plate. Technicians operate the bank every day and it is a simple matter to "dip the tanks" and make any necessary adjustments. This further adds to the value of the method since someone has a regular task to inspect each storage vessel every day.

Because of the amounts of liquid nitrogen in store at any one time it is important that all staff operating in the area have read and had explained to them the safety requirements for handling cryogenic liquids. (see appendix). As an additional safety feature we obtained through BOC a continuous monitoring Teledyne oxygen sensor and audible

alarm which operates at all times in the work area, being relayed to a central area. It is set to give a danger signal at 19% oxygen in the inspired air in the event of heavy nitrogen gas displacing oxygen due to excessive gas leakage. The alarm probe is set at chest height so that adequate warning of irrespirable gases would be given. This system has never operated under such extreme conditions but occasionally signals during times when the arrival of liquid bulk nitrogen supplies are being pumped from the road tanker into our storage vessels on days when the wind is able to cause a swirl of gas into the work area. This is usually a sign for the technicians to close the outer doors and evacuation of the work area is not necessary.

Storage of Sub-samples

As described the method of storage with the main donation is convenient and practicable. For storage of samples in clinical frozen cell banks Rowe et al (1965, 1967) used a method of spraying droplets of blood mixed with 0.3M glucose + 0.45M sucrose as cryoprotectant on to a revolving cone of liquid nitrogen. Storage of the "blood sand" was at -196°C and recovery in 0.15M saline at 37°C. There was full retention of 70 different human blood group antigens and the enzymes glucose 6 phosphate dehydrogenase, lactic dehydrogenase, glucose-pyruvate transaminase, glucose-oxalacetic transaminase and aldolase were unimpaired. Rowe et al (1970, 1971) reported this as being their method and stated that it required a separate inventory to the main donation and is as inconvenient as the original method described by me. De Verdier (1965) used small flat aluminium foil containers each holding up to 12 ml for some

original pilot studies in the low glycerol rapid freeze technique and from this the larger flat steel and corrugated metal cans were developed by Rowe and others who later described a method of freezing the integral segmented tubing from the original glycerolized blood pack to be stored in a special compartment alongside the main container. Baar using hydroxyethylstarch as cryoprotectant froze small volumes of blood in small aluminium containers as did Bowman et al (1973). Mohn (1968) used steel containers for up to 3.5 ml on a low glycerol liquid nitrogen system. Huggins (1970) used vacutainers of 2.5 ml capacity which were frozen with the main pack at -80°C requiring washing with six different wash fluids before use in matching tests. Akerblom and Hogman (1970, 1974) used Dextran 80 as cryoprotectant with droplet freezing in liquid nitrogen. Later with the development of their cryostable Kapton FEP bag a separate compartment was constructed in which to hold sufficient cells for matching tests.

Reid and Ellison (1974) reverted to droplet freezing in a multisegmented metal tray filled with liquid nitrogen into which cells in sucrose/dextran could be dropped. It can be seen that not all workers keep the donations and the pilot samples together and that very often they are each prepared by entirely different techniques and each has its own different recovery process. To avoid this my original method referred to in the reprint was developed. Initial studies showed that as well as using the residue of glycerolized cells, the same wash solutions in the same order could be used for recovery, a frozen aliquot being broken into a McCartney container of the first wash solution (10% sorbitol) followed by centrifugation, decantation and

resuspension in normal saline with repeat wash (second) to give a red cell suspension more than adequate for any matching tests. Red cell antigens were well preserved (ABH, CcDEe, MNs Kell, Duffy, Lu^a, Lewis, Kidd and P1, Kp^a, Kp^b, Cl^a). At least 50% recovery of red cells can be easily obtained (see Appendix). The residue of the glycerolised cells remaining in the donation bag at the initial can filling procedure is recovered and pipetted into each of five polypropylene tubes each capable of containing 0.4 ml blood glycerol mixture (equivalent to 15 drops from a Pastein pipette of gauge 16 size). The method was compared with a standard PVP drop method after processing by the technique given in the appendix with no evidence of any loss of antigenicity in the ABO, Rhesus, P, MNSs, Kell, Duffy, Kidd, Lewis systems. Other systems have been tested as reagents became available and these include Caldwell, Ii, Wright, Kp^a, Kp^b, C^w, C^x.

Since the original introduction of this method the system of freezing and recovery has been used thousands of times without any difficulty or anomalous result.

Method of Thawing

Although the flotation collar system works perfectly well for thawing out single units of frozen cells problems of space arise when two or more units are required. When the author visited the Netherlands Red Cross Transfusion Centre in Amsterdam, Krijnen and his colleagues, although rarely using frozen cells, (see report) had had a few unpleasant experiences with storage cans explosively rupturing due to small amounts of liquid nitrogen trapped at the time of freezing or subsequently (Jenkins and Blagdon 1971). This had prompted them to adapt a simple agitator clothes washing

machine to carry cans during thawing. With the assistance of the Area Manager of the South West of Scotland Electricity Board some obsolete domestic washing machines were obtained as a gift (the manager is a haemophiliac!). These were of the central paddle agitator type and have a solid lid which was fitted with retaining clamps so that in the event of a can rupturing or the stopper blowing out the shock impact is absorbed. Initially the cans were placed in metal carriers slung from the side of the metal casing and the bath water at 40°C made to move quickly at about 100 cycles per minute. This proved effective but cans tended to be only moved from side to side. It was decided to increase the agitation by attaching them to the central spindle as shown in the reprint of my paper to J. Clin. Path. (1972). This ensured that up to four cans could be thawed simultaneously. The system has proved successful and suitably adapted "gifts" from the Electricity Board have been "exported" to Edinburgh and the idea taken up by other Centres who have visited us. There is no doubt that sometimes violent pressure rupture of cans does occur despite the most careful wiring of the special sub-a-seal closures (see Appendix) and avoidance of splashing during the freezing process (see Plate 7/10). Similar explosive rupture has occurred in other places (Guy's Hospital, Army Blood Supply Depot, Bristol R.T.C.). This is the reason that technicians like the idea of the heavy lid over the top of the cans during thawing. In an effort to minimize this risk a skirt of paper was constructed to point downwards and keep the neck of the cans dry during freezing. This was awkward to handle and recently we have now started using a standard aluminium foil cover which can be moulded around the neck of

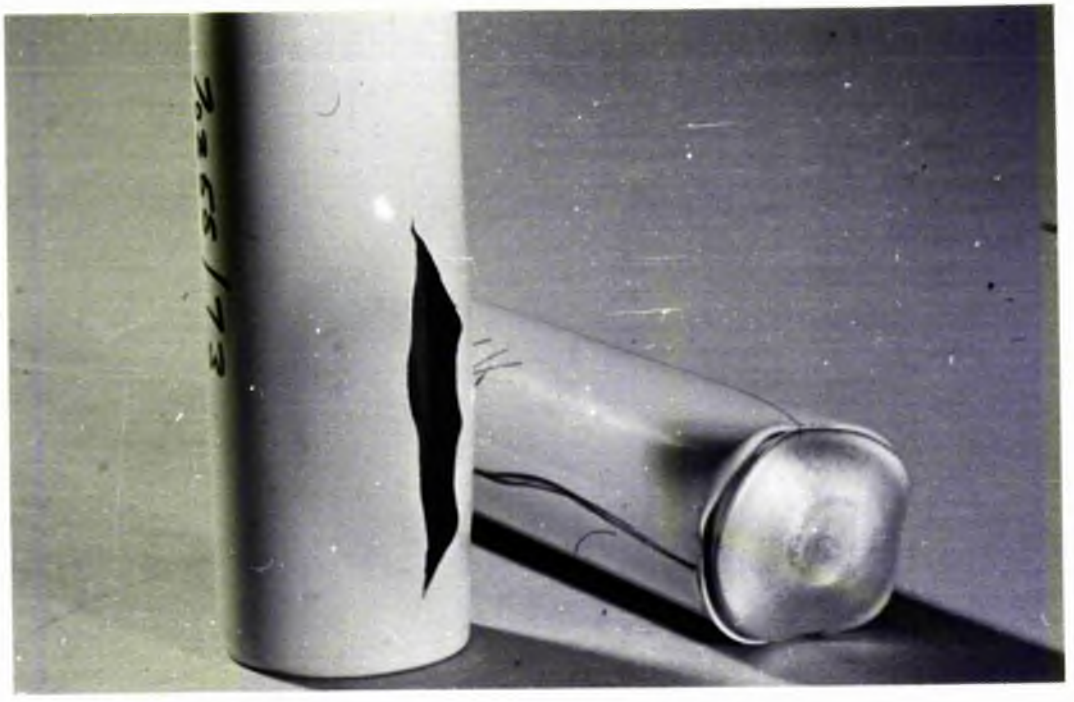


Plate 7/10. Effects of Pressure Rupture

the stopper prior to freezing and storage. During freezing the foil shrinks tightly over the rubber stopper and dust cap. So far the system has worked very well and there have been no more ruptures during thawing. If this is confirmed then it will be most valuable since pressure rupture is given as a reason for some workers to change over to or adopt freezing methods which rely on cryostable plastics (Rowe et al 1970, Akerblom, and Hogman 1970, 1974, (volume 1) and was the original reason for the gas tight specially engineered can closure in Krijnen method (Krijnen 1970). Needless to say these "special" freezing containers are much more expensive than the standard Finnish "Printaloy" can developed by Jenkins and Blagdon, who quoted a price of about £1-50 per unit processed complete compared to the present cost of cryostable plastic bags are about £3 per bag which does not include running and processing costs (p 116).

Why not freeze all blood?

Putting aside the question of costs for the moment some might argue that frozen blood has certain advantages which might make it desirable to stockpile large quantities of blood which could end the outdating of conventional stocks once and for all, which could avoid the problems of hepatitis very largely and make transfusions safer by avoiding sensitisation and allergic reactions of the non-haemolytic type associated with leucocyte, platelet and plasma antigens unwanted cations and cell debris. Bryant and Wallace (1974) considering the evidence stated that frozen erythrocytes provide a superior component as compared to conventionally stored cells. They were, like most U.S. blood bankers, very concerned over donor availability and the vulnerability of donor intake to seasonal fluctuation due to the features

already noted (p 98).

If one were to process 100 units per day with an average storage of say, 3 months than for one years supply 9000 units of blood are required. To cope with an annual intake of 100,000 donations (e.g. West Scotland) would need 25,000 units of storage space, assuming a 3 monthly turnover. For a 6 monthly turnover, the more desirable, one would need vivostat capacity for 50,000 units! Since each vivostat holds about 200 units of blood then we would need 250 vivostats with the liquid nitrogen and staff to fill and service them. What is needed is the means of removing or neutralising (inactivating) hepatitis virus and a means of purifying blood of its leucocytes, platelets and other components which can be removed for component therapy programmes. These steps, if ever developed, might then reduce the requirements for a frozen cell bank to very reasonable proportion for the transfusion of patients who cannot be transfused by conventional means (rare donors, rare recipients, auto transfusions). Thus Ogden's (1972) approach to blood transfusion was to try and eliminate the need for transfusion! It seems that that day is not yet near and it is likely that further expansion will occur in USA if not UK and other places quite outside the military considerations for stockpiling for mass civilian casualties and military conflict. This requirement is likely to be for patients who cannot tolerate ordinary red blood cells. Tullis and Lionetti (1966) describing the use of frozen blood for autologous transfusions estimated that about 40% of all transfusions of conventionally banked blood was used for non-emergency and predictable reasons of elective surgery, obstetrics, pre-operative and post operative anaemia and red

cell [illegible] deficit medical conditions. Such a large number of transfusions could be planned and done with frozen autologous cells. They made the novel observation that if an adolescent were to give one pint of blood each year to a frozen cell bank during his young healthy years, the great majority of non emergency transfusion needs could be fulfilled later in life. In addition to ensuring an adequate supply of blood all recipient reactions would be abolished, there would be no allosensitisation, serum hepatitis or other transfusion transmitted disease. The patient would receive only those diseases, immune globulins, haptoglobins, haemoglobins and other group specific materials which already are uniquely his own. The feasibility of such a programme however presents an enormously difficult set of technical and storage problems. Nonetheless as early as 1962, Haynes and Tullis (unpublished - see Tullis and Lionetti 1966) demonstrated that autologous transfusion of an unusual group could be used to perform successful cardiac by-pass surgery.

On a small scale Becker et al (1971) used 100% frozen blood in a small community hospital requiring 1300 units per year. The average cost of processing frozen blood was 26 U.S. dollars in 1970 (Huggins method) 100% freezing released valuable platelets and plasma fractions including cryoprecipitate which, they calculated, added a further 13 dollars to the total cost. They took into account non payment to a volunteer donor, costs of plastic bag, glycerol, reconstitution sets, glucose and wash solutions, technicians time, capital equipment, maintenance and electrical factors. The costs of this small venture were carefully worked out. The costs of equiping and running a frozen cell bank are often exaggerated and fade into insignificance when compared with the cost of modern automated haematological equipment used in most large laboratories or the cost of a home dialysis programme. Patients who get transfusion transmitted jaundice pose a threat to other patients and their attendants and one must remember the cost of in patient care in the present economic climate (Lewis et al 1973; Szmunessal et al 1974). The costs of some types of frozen cell bank are quite expensive although the methods described in this thesis are probably the cheapest yet available. The many indirect savings such as decreased personnel time involved in investigating allergic and febrile reactions, tracing donors involved in alleged hepatitis cases are difficult to measure but nonetheless important.

Many transfusionists still have reservations about frozen erythrocytes (Chaplin 1974):

1. There are too many procedures which means they are not much good and should await the development

of a final or alternative technique.

2. The techniques are too esoteric and complicated.

3. Since extensive education is required to encourage physicians to use components they will be very reluctant to accept the use of frozen cells without a tremendous effort on the part of blood bank Directors.

Some of these criticisms are no longer valid. It is true that just as the 1950's marked the early discoveries of freeze-thaw injury and its prevention, the 1960's the development of a multiplicity of methods aimed at achieving cryopreservation of bulk samples of blood, the 1970's so far have seen the emergence of only a few methods which are now well established and which offer feasible reproducible methods easily taught to technical staff and able to guarantee a clinically acceptable product of superior physiological function free from unwanted cations, plasma proteins, platelets, white cells, microaggregates, viruses and bacteria. The methods are not yet perfect but if we wait for the universal adoption of the ultimate method we might just as well wait for the adoption of a perfect universally accepted method to perform so many other tasks which are performed every day such as compatiblility testing, antibody detection, counting foetal cells, bilirubin estimations etc. There are in fact only three major methods from which to choose - the Huggins system with its high glycerol, slow freeze method with either cytagglomeration or continuous counterflow centrifugal washing for deglycerolization (the Meryman method) and the Rowe (Krijnen, Pert) method with low glycerol rapid freeze at -196^{o}C with deglycerolisation by either continuous flow machine washing

(Haemonetics) or batch (serial) centrifugation - the method of choice in the present thesis.

So far as rare blood storage or military use is concerned, Valeri (1966) states:- "Whatever the cost, it is justified when the need for rare blood types creates a transfusion problem, or in the advance planning for a disaster in which regular blood banking facilities would be inadequate." Cost accounting for the frozen blood product includes expenses of equipment, materials and labour. In addition non-monetary costs must be considered. In the logistics section (p 110) consideration of the desirability and feasibility of massive storage has been given. Recent heated discussion on the costs of frozen cells has appeared. Moss et al (1974) hold that the costs are justified whilst Chaplin et al (1974) take the opposite view. Our own experiences will now be recounted and reference made to the published costs of other workers.

Since the frozen cell bank has been subject to change and increasing commitment from its beginning in 1971 it will be useful to trace how this has reflected on the economics of running such a system.

Initially the Scottish Home and Health Department Grant £3,000 was for a feasibility study into the setting up of a bank based on the application data shown in the appendix. At that time the author had studied the available system and manufacturers equipment (British Oxygen Co., Union Carbide Co. and Spembly Technical Products). A comparative table was drawn up to show what was available for each manufacturer.

Store Model	Union Carbide	Spembly	B.O.C.
L.R. 250	£1300		
LNFS 125		£1350	
CPV 250			£695
Freezer Unit			
L.R. 40	£266-10		
T/DLNF 10		400	
ODS 18			30
Top up Storage			
L.S. 1603	361		
TWN 200/2 Supply Tank		not avail.	567
Level indication gauge	not available	" "	23
Pump for Tank	" "	60	92
Level alarms	" "	60	37
Totals	£1927-10	£1910	£1414

From these costings it was decided to purchase the B.O.C. equipment since it was (a) cheaper, (b) just as good as a

liquid nitrogen refrigerator made to high standards of superinsulated containers in a high quality steel skin, (c) it was compatible with all other equipment in the form of a package deal, and (d) certain items such as pumps and level sensing alarms. Since the B.O.C. vessel can hold approximately 160 cans of blood and allowing a 3 monthly turnover of stock the cost per year in the first year for freezing and storing a can of glycerolised blood is £1414 ÷ 4 x 160 = £2.209. The equipment has a life of at least 10 years so that one can deduct a figure for amortization say, 10% per year for 10 years. This brings the price to about £2.00 extra on top of a conventional unit of blood as capital cost.

Jenkins and Blagdon (1971) quoted the cost of their system as being £2,500 to set up a bank of 500 units i.e. £5.00 capital cost. These take account of the cost of the soft ware which account as follows:(1975 prices)

		Unit cost
Metal can	£264/1000	0.264
Subasealclosure cover		0.060
Subasealclosure		0.066
Pilot can	£20.24/1000	0.0204

To this, one must add technical time and assuming approximately half of the time is spent in liquid nitrogen work we have:

Chief Technician	@ £2.25 /hour	£4455 / annum
Lab. Assistant	@ £0.82 /hour	£2032 / annum
Cost of manpower	@ £3.07 /hour	£6487 / annum

Assuming 1000 units of blood stored and processed every year,

cost of manpower per unit of cells £6.487 / unit

Assuming that roughly half of their time is spent in freezing down cells, keeping records and maintaining equipment and supplies and that the other half is spent recovering cells

from the frozen cell bank, preparing them for patients, keeping records and stocks of recovery equipment, the unit cost for the freezing process is: £2.03/ unit

On the Recovery the cost analysis is:

Cost of	5 Tail bag for deglycerolisation	£ 1-54
	Sorbitol wash/unit (250 ml)	0-02
	Saline/unit (50 ml)	0-02
	Bacteriological reagents for quality control	
	Haematological testing for quality control	
	Transfer line (Fenwal AE 9)	0.10
	Airway needle (reusable)	
	Manpower (½ available time allocated)	1.621
		£ 3.301.

Jenkins and Blagdon (1971) calculated the running costs of their bank inclusive of labour and materials, but excluding the primary pack since this is always needed in any system (otherwise no donation is collected at all!) was approximately £1-50. Our own costs would agree with this. More recently of course, inflation in the economic sphere has increased all of these costings.

In our system the total amount of liquid nitrogen required per year in 1972-73 was approximately £1000. This works out (with a 1000 cell bank/year) as £1 per unit and this is a non recoverable loss into the atmosphere. (The nitrogen is needed for freezing the donations and maintaining the temperature in the vivostats).

With the growth in red cell freezing the nitrogen usage is increasing each year. Since the beginning we have been obtaining it on a central Ministry of Technology contract with B.O.C. which at the time of writing is £0.1193 per litre delivered to our vessels by road tanker.

Gradually we have increased the size of the bank until we now have eight vivostats and four L.R. 40's, two TWN 500

litre storage tanks, four pumps, one laminar flow sterile air cabinet and satellite banks of equipment are operated by Glasgow Western and Glasgow Royal Infirmaries.

Cost accounting for the frozen blood product includes expenses of equipment, materials and labour. In addition non monetary costs must be considered. Thus the time involved in preparation of the transfusion unit may be of critical importance (Valeri 1969).

Haynes et al (1960) calculated the total cost of their high glycerol slow freeze (-80°C) and recovery in an automated centrifuge to be 14 dollars per unit. This only included the disposable bags, tubing, solutions and labour involved in processing, storage, reconstitution and final preparations. Allowing the costs of other equipment and amortization over 5 years and in operational level of 3000 units per year, the overall cost was 16.40 dollars.

Perrault et al (1967) calculated the cost of processing one unit of blood from a high glycerol -80°C bank as 20 dollars. This included only the freezing bag and wash solutions and did not take account of the capital cost of the processing unit (cytoglomerator 10,000 dollars) and the mechanical two stage refrigerator (3300 dollars).

Commenting on the costs of frozen cell banking found in response to a questionnaire sent to laboratories in U.S.A. and Europe. Chaplin (1974 a,b,c) made some interesting comparisons. The costs varied from one centre to another since there was no uniform way of accounting. Some differences were due to different techniques are probably truly reflected valid comparison, others did not include the costs of initial capital equipment and amortization.

Centre	No. of frozen units transfused	Estimated Cost Frozen	Liquid
Brentwood, U.K.	647	£ 4.0	£ 1.7
Uppsala, Sweden	21	$46.0	$14.00
Amsterdam, Netherlands	40	$18.8	-
Ottowa, Canada	30	$37.26	-
Bethesda, U.S.A. NIH	12	$61.0	$ 7.50
Arizona, U.S.A. Red Cross	50	$54.20	$18.0
New York, U.S.A.	1674	$38.00	$17.50
Philadelphia, U.S.A.	333	$60.00	$30.00
San Francisco U.S.A.	199	$51.38	$21.60
Chicago, U.S.A.	431	$66.30	$33.0
Boston General, U.S.A.	7000	$50.55	$30.0
Boston, Red Cross U.S.A.	1800	$43.68	$16.68
Boston, Deaconess, U.S.A.	1552	$84.20	$25.0
Boston, Chelsea, U.S.A.	1000	$20.40	-
Becker et al (1971) U.S.A.	1300	$26	-
Present Work(1975) U.K.	1000	£ 8.55.	-

Costs of Frozen Blood (Chaplin 1974)

Table 7/2

Costs of Frozen Blood Compared.

some did not include the cost of additional technician time. His survey (Table) applies to 1970-71 so that it is possible to compare this with our own calculations, although costs have (like others) increased markedly in recent years. Pepper (1976) has estimated that his costs in Edinburgh are £3 - 4 for manually produced and £6 - 12 for automatically (machine) processed blood (£8 in 1974! for freeze, store and recover excluding labour).

One thing is clear from these cost comparisons even in centres with the largest usage the cost per unit is 2 - 3 times the cost of a unit of conventionally stored liquid blood. To store large numbers of donations would add considerable expense. Chaplin (1974) calculated that freezing the U.S.A. intake would add about 200 million to 400 million dollars to the cost of health care. Nevertheless Mitchell (1976) writing on the optimal use of blood as a scarce national resource showed the vast wastage which occurs each and every year. Applied to the U.K. these arguments would be that if 25% of the donations voluntarily given each year out of a total intake of 2 million were to become outdated and not used, then assuming a cost of £5.00 per unit then the wastage in economic terms becomes £2½ million. All of this red cell component is totally wasted and if it were not for the fact that the plasma may be used for fractionation, might just as well not have been collected. Even more importantly it represents a wastage of the <u>entire circulating blood volume of 50,000 able bodied adult men each year and every year</u>. My thesis is that at least some attempt should be made to store such resources in frozen cell bank at least and until there is a more enlightened usage rate by clinicians. Appeals to the public to volunteer as blood donors are not

likely to have much additional impact in the face of such figures. The use of computers, improvements in liquid preservation may have some effect (Mitchell 1976) but there is room for considerable advance by these and other means.

In Summary the costs are (late 1975):

For freezing	£2.031
For recovery	3.301
For nitrogen	1.00
For storage (over 10 yr)	2.00
	£8.332 /unit/10 yr

CAUSES OF FAILURE

Haynes et al (1960) listed the causes of failures in his frozen bank. Most of these occurred at the beginning of their work in 1956 and represented about 3% of their turnover. Two hundred and twenty units were unsuitable out of 1471 successful recoveries due to bags leaking, low yield, personnel error, equipment breakdown, autoclave breakdown, defective water supply, contamination of resuspension media and excessively high haemoglobin in the supernate when using commercial saline of abnormal acidity. As experience was gained the loss rate from all causes was reduced to less than 1%. Throughout the present study our experiences have almost paralleled these observations. Most problems were solved in phase 1 (8:11:71 - 31:01:72) and the problems of phase 2 (1:02:72 - 31:01:73) were mainly due to the familiarisation needed when new workers had to be taught to do the recovery work. Early in phase 2 considerable difficulty was experienced at Glasgow Western Infirmary in that inexplicably high supernatant haemoglobin levels were common in the recovery washing sequence until it was discovered that commercial saline of pH 5.6 was being used in preference to the saline at neutral pH manufactured by the Regional Transfusion Centre. Such poor recovery results were obtained that serious thought was given to abandoning phase 2 and reverting to all frozen blood being processed at the Regional Transfusion Centre. Units of frozen cells transported to and from the Western Infirmary could be recovered at Law without any difficulty and yet local recoveries under supervision of Law staff in the Western Infirmary Laboratory resulted in poor results despite meticulous attention to detail. This suggested that (a) the

cells were being delivered in good order and that the error was during the subsequent recovery washing period. Due to the persistence and encouragement of the Consultant Haematologist Dr. H. Hutchison, the problem was resolved when a change to neutral saline was made.

APPENDICES

Relevant papers are included in this appendix to which reference is made in the text. The reports represent only a small portion of the technical memoranda written by me and are used to illustrate to debt which I owe to so many individuals who have given so much of their time and energy to make this project worthwhile.

"The moving accident is not my trade;
To freeze the blood I have no ready arts:
'Tis my delight, alone in summer shade,
To pipe a simple song for thinking hearts".

Wordsworth, Hart-leap Well
pt. ii st. 1.

APPENDIX 1

VISIT TO BRITISH ARMY BLOOD SUPPLY DEPOT

Glasgow and West of Scotland
BLOOD TRANSFUSION SERVICE

TELEPHONE NOS. WISHAW 73315/8
TELEX NO. 77963

REGIONAL DIRECTOR
JOHN WALLACE B.Sc., M.D., F.R.C.P.G., F.R.C PATH.

AT LAW HOSPITAL,
CARLUKE,
LANARKSHIRE.
ML8 5ES

RM/MC

24th February, 1970

Major Derrick Robson,
Army Blood Supply Depot,
Ordinance Road,
ALDERSHOT.

Dear Derrick,

I am writing to thank you and Colonel Munro for your kindness in allowing me to visit you on 17th February. I am deeply grateful to you for the care you took in explaining to me some of the problems of cryobiology.

As I indicated to you when we were at the Station, I would put my thoughts in some kind of order and discuss them with Dr. Wallace. As a preliminary, I would be grateful if you could comment favourably or unfavourable on the following:

1. As I see it, there are two basic low temperature systems (i) quenching at minus 80°C with storage at the same temperature in a mechanical deep freeze cabinet; (ii) quenching at minus 190°C in liquid nitrogen and storage in liquid nitrogen.

2. To achieve this a cryophylactic agent is required. These are (i) glycerol, high and low concentration and (ii) polyvinylpyrollidon, (iii) hydroxy ethyl starch.

3. To be effective at the moment we could use the low glycerol minus 80°C technique with reconstitution after storage using either a modified Huggins' matching technique as being developed by Fenwal (Baxter's) or a continuous (and quicker) centrifugal washing technique also being investigated by Fenwal. This latter method is probably not suitable at the moment but the first alternative should be possible provided Baxter's can supply ex stock washing equipment. We could certainly make our own solutions.

In this way we would have a ready clinical capability in a short time since we have ordered and should be taking delivery soon of a minus 80°C deep freeze cabinet. The costs involved would be: Staff, Baxter's unit (£100). Baxter's washing equipment (cost unknown), minus 80°C storage cabinet (bought), washing solutions (made by us at cost).

4. To be effective in 2 - 5 years time, we could think of a

liquid nitrogen technique. The methods available at the moment are trying to achieve (i) long storage beyond 2 years; (ii) easy washing or no washing. Item (i) hardly concerns us since storage to 2 years might be adequate. Item (ii) is a dream and is unlikely to be realised.

5. By combining actions 3 and 4, probably offers the best of both basic methods. The system most likely (in my view) to succeed will be:

Having developed a suitable closed system of container to hold donor blood, initial separation will be done of cells and fresh plasma. To the cells will be added a cryophylactic agent and the whole unit then quenched in liquid nitrogen. Thereafter storage in liquid nitrogen and recovery beyond two years (N.B. any such experiment started now cannot be completed until 1972-73!)

The recovery process will probably require multiple washing. This combined system again will probably use either modified Huggins but more likely continuous centrifugal washing techniques. These techniques are not yet developed sufficiently to enable an early clinical application.

In conclusion therefore of the three systems (3, 4 and 5) stated, number 3 (low glycerol minus 80°C modified Huggins wash) is ready for clinical use and exploitation; number 4 (liquid nitrogen) is more costly and less dependable with more "snags"; number 5 (combined low glycerol liquid nitrogen, continuous centrifugal wash recovery) is a good compromise.

I think therefore we should:

(i) Establish the low glycerol, mechanical deep freeze (minus 80°C) technique with modified Huggins wash recovery routine.

(ii) Having obtained experience with this in the intermediate handling of equipment and reagents, develop as a research interest, initially, and a clinical capability ultimately, the liquid nitrogen techniques.

(iii) By the time we would be ready for liquid nitrogen on a large scale to replace the minus 80°C some of the snags would be solved such as:

(a) containers

(b) wash recovery routine (- Baxter's)

(c) cryophylactic agent

(iv) As a research interest we might keep watch on these developments in the liquid nitrogen field and perhaps make our own observations concerning other products. In particular I have in mind the supply of platelet concentrates and their long term storage. Platelet concentrates are usually in 10 - 20 ml each and might be suited to laying down in such small volume as distinct from the 500 ml volumes of red blood corpuscles.

I look forward to hearing from you soon and welcoming you to Scotland if you get a chance to come this way.

Kind regards,

Yours sincerely,

APPENDIX 2

APPLICATION FOR DEVELOPMENT GRANT

PROLONGED PRESERVATION OF BLOOD AT VERY LOW TEMPERATURES

Application for Development Grant by West of Scotland Blood Transfusion Service, at Law Hospital, Carluke, Lanarkshire. ML8 5ES

27:3:70

INTRODUCTION

It is well known that in large transfusion centres, stocks of available blood fluctuate throughout the year. This is due to a number of factors such as:

(a) Unexpected demand for whole blood and whole blood products. e.g. Major accidents, surgical emergencies, platelet transfusions in childhood leukaemia.

(b) Anticipated but special demand for special products, e.g. Open Heart Surgery.

(c) Natural seasonal fluctuations due to long regional industrial holiday periods with consequent shortage of donors.

(d) Cancellations of donor sessions at short notice due to strikes and similar industrial actions at large factories.

(e) Sudden epidemic illness, e.g. influenza.

(f) Shortage of rarer blood groups.

To try and alleviate these shortages, transfusion centres have embarked on schemes for the long term storage of blood. By "long term" is meant the storage beyond the usual limits achievable by conventional chemical methods of preservation (usually 21 - 42 days). These long term methods involve suspending the red blood cells in some additive (usually glycerol) and preserving the suspension at very low temperatures either mechanically or by the use of liquid nitrogen. Such practical methods for the frozen storage of red cells are already available. These have been used clinically but much remains to be developed notably improvements in the methods of glycerolisation and deglycerolisation of cells.

Counter-flow centrifugal washing procedures are being developed at the moment and these will improve speed of recovery of frozen cells.

ADDITIONAL ADVANTAGES

In addition to the advantages of a long term store of cells to stabilize the fluctuations already referred to, there are other more subtle and perhaps far-reaching scientific reasons for long term preservation.

(a) Stored and washed cells should be ideal for use in connection with transplantation problems - beforehand to avoid isoimmunisation by leucocyte, platelet antigens and plasma factors; afterwards to avoid stimulation of the graft rejection mechanism.

(b) In the treatment of chronic refractory anaemias to avoid isoimmunisation. eg. Thalassaemia, Aplasia, Hypoplasia.

(c) There is some evidence that the use of frozen cells may reduce or almost eliminate the occurrence of post transfusion hepatitis and other transfusion transmitted diseases.

PROPOSITION

It is proposed to set up in the West of Scotland Regional Blood Transfusion Centre a long term store of human blood.

1. Using existing equipment to store at minus 80°C in a mechanical deep freeze unit. This has the disadvantage that it may be unacceptable for storage of large amounts because of the risk of mechanical breakdown. Another reason is that recovery and deglycerolisation is more complex and commercial interests are not pursuing an active policy in trying to improve the recovery procedures. Nevertheless, for experience in low temperature work and the storage of other blood products especially plasma products and platelets, such a development is desirable.

2. To purchase and commence using methods of preservation using liquid nitrogen. It is clear that this is the most rapidly expanding method of preservation. It has been tried in various centres and shown to work. It has several advantages.

(a) It is non-mechanical and therefore less liable to failure.

(b) Commercial organisations show rapid advances in the field of recovery and counter-flow centrifugal washing procedures.

(c) We have been using liquid nitrogen storage in a small scale for some years now in the preservation of rare blood group cells.

(d) Lower concentrations of glycerol are added and therefore less has to be removed at the time of reconstitution.

AVAILABILITY

Two major organisations manufacture liquid nitrogen storage equipment.

(a) Union Carbide (U.K.) Ltd. This Company make the large variable size Dewar flasks. These vary in capacity and are available soon after order.

(b) Spembly Technical Products Ltd. This Company has long experience in cryobiology and are at present producing special order equipment consisting of all the basic parts required to freeze and then store red cells.

We have seen the representatives of both of these firms and feel that (b) is the better proposition because of knowledge of cryobiology especially blood preservation. With their basic system, additional storage units of variable capacity can be added later.

Thus it is proposed that a small basic unit be set up initially to process up to 250 donations. This would be developed as the project progressed.

COSTS

To establish the liquid nitrogen processing and the liquid nitrogen storage as stated would cost in the order of £3,000. Maintenance costs would be about the same as for mechanical refrigeration stores. Storage space can be made available at Law and in a new proposed processing laboratory extension which is already allocated. Existing staff would be allocated to the project.

ADDITIONAL RESEARCH AND TEACHING

In addition to research experience gained by using the equipment, we would propose to investigate its use in the preservation and storage of other formed elements of blood. The teaching of technical, under-graduate and post-graduate medical personnel presents a great opportunity to educate those interested in the uses and availability of such a system of preserving tissues.

APPENDIX 3

VISIT TO BRITISH OXYGEN COMPANY

VISIT TO BRITISH OXYGEN COMPANY - 7:10:70

Following my letter to Mr. Wild of Spembly Technical Products and his visit to us on 30:9:70, I decided to see the B.O.C. range of equipment at their Open Day in London. I specifically wanted to clarify the following problems.

A. To ask about the CPV 250 Mk 2 Vivostat: I saw this equipment and discussed it with B.O.C. European Exports Manager (Mr. Reed), Mr. Snowman (U.K. Technical Representative especially concerned with biological problems). Dr. John Blagdon from Brentwood was also present during these discussions and heard both my questions and B.O.C. replies:-

Q.1 Why is B.O.C. CPV 250 costing £70[illegible] and Spembly and Union Carbide equipment of the same approximate dimensions 100% more expensive (£1,300).

Ans. B.O.C. were surprised that their competitors were so different. They said that they batch manufacture in U.K. rather than make to customer specification and, of course, have no import duty. Their prices are similar to the prices paid for other competitors (French) products on the continent of Europe.

Q.2 What sort of guarantee would B.O.C. give regarding faulty workmanship, faulty design, sudden or gradual loss of vacuum.

Ans. B.O.C. are too big a company to be selling poor quality materials on the British market especially since their troubles with the Monopolies Commission 8 years ago. They are unlikely to put such an item like the CPV 250 on display at an Open Day if it was thoroughly bad. They started and have maintained production of the CPV 250 for 2 or 3 years and have had no major trouble as we described. They do admit to an occasional loss of vaccum and say this can happen with any competitors equipment as well as B.O.C. Inevitable leaks through the shell occurs over the years. We were then joined by B.O.C. Head Workshop Foreman who

assured us further that he had occasionally had to pump down a new vacuum for customers' vivostats used for bull semen. He knows of one sudden failure of vacuum where their mobile units were able to copy. Specifically then the warrenty would be:

The vacuum could be expected to hold for one year from the date of purchase. Note that the vivostat might have been manufactured earlier than this. In the large majority the vacuum might need repumping every 3 years. B.O.C. could probably arrange (a) immediate delivery of spare vivostat from Newcastle using our own transport, (b) a possible insurance/ maintenance contract for regular servicing, (c) equipment on a "hire plant" basis at approximately one third of the purchase price per year to be purchased over 3 years. Thereafter there would be a small service charge. This last scheme does not appeal to me since we would not own the equipment.

Later in these discussions we were joined by Mr. McKechnie, the West of Scotland Gasses Division Representative. He stated that as far as he was concerned he would personally see that we did not lose refrigeration storage or capability in the event of failure of a vivostat or other supporting system.

Q.3 How many cannisters of the Helsinki design could one store in the CPV 250 vivostat.

Ans. (Mr. Snowman). Probably 150 but they will check with their drawing office and let Dr. Blagdon and I know.

Q.4 What system of storage would be used.

Ans. (John Blagdon). Two teir system of two cannisters one on top of the other inside tall cylinders resting on a perforated aluminium base plate with liquid nitrogen level about half way up the lower cannister. The base plate divided into quadrants to make filing and retrieval easy.

Q.5 Could level alarm be fitted to the lid.

Ans. Yes, easily, by passing it through the split lid. Automated top up could also be done this way.

Q.6 What is the long term intention of B.O.C. in the cryobiology field. In 10 years (subject to widespread design) what are the chances of obtaining spare parts.

Ans. (Mr. Reed). B.O.C. are in the market to have no foreseable plans to pull out.

B. To Ask about Collateral, Back up and Aftersales Service

This conversation took place between myself and Mr. McKechnie.

B.O.C. would be willing to send a Customer Relations Representative to advise regarding the suitability of the various sites at B.T.S. for locating equipment, storage and access for bulk tanker delivery of liquid nitrogen. I have arranged for this to be done sometime in the week commencing 26th October, 1970.

B.O.C. would be reluctant to give us tanker delivery for a 200 litre storage tank but following a telephone call between Mr. McKechnie and Glasgow I was given an assurance that this would be done without prejudice to the vivostat system which we might purchase. Obviously B.O.C. would prefer us to have a 500 litre tank and buy all vivostats from them.

B.O.C. would sell to us at the central contract price for liquid nitrogen. At the beginning of installation B.O.C. would fill the vivostat(s) to the required levels, fill all supporting storage tanks and dewars. Thereafter tanker delivery would be on a regular basis depending on the rate of usage.

I explained to Mr. McKechnie that compared with industrial uses of his products we were a very small organisation and therefore could not offer a large investment return for these back up services. Nevertheless he appreciated the value of our products in human terms and the guarantees which we must have against failure of our storage

system.

Filling and draw off systems were discussed. We would use the wheel mounted horizontal TWN tank with liquid nitrogen impeller pump and hose attachment. All of these work at atmospheric pressure. High flow rates can be achieved using pressurised systems but these are not considered necessary since we would be only "topping up" from the main tank and drawing off small volumes (at 3 litres/min). The newer TWN storage tanks have a long tube filler which reaches below the level of liquid nitrogen remaining in the tank. About 9% loss by "boil off" can be expected but B.O.C. would charge us only for a full tank and not what boils off in venting the flow lines.

C. To look at the new cold cabinet in which Dr. Ibbottson (Birmingham) is making cryoprecipitate

I met Dr. Ibbottson and saw the type of processor involved. I have details of this but it is not relevant to this Paper.

D. To meet and talk with Dr. John Blagdon

Following my letter to him of 7:10:70 he had decided to visit this Open Day at B.O.C and we talked privately about a number of things.

He is now shopping like us for equipment. He hopes to have the final go ahead on his grant from the Ministry (£19,000) at the end of this week. He intends to buy 4CPV 250 Mk 2 Vivostats together with support systems. (He also cannot reconcile the low price of the B.O.C. Vivostats). He is including in the grant moneys to pay an S.R. Technician and a part-time secretary to look after the filing of documents laid down. Also included is money for a new SP 600 Spectrophotometer to do enzyme studies. They will also do stereoscan electron microscopy of frozen red cells.

He is still a little reluctant to commit himself irrevocably in these matters of buying equipment but I think if we move he will do the

same.

We discussed also recent textbooks on frozen blood, the attitude of Mr. Wild of Spembly to supplying cannisters, subaseal closures and gas tight seals on these cannisters, pilot tubes, sources of glycerol and sorbitol, his experiments on the new cannisters, availability of washing bags from Dr. Krijnen, Avon washing bags, date of publication of his Paper with Professor Stewart, Dr. George Stoddart's project at Guy's.

Conclusion

I think we should go ahead and place firm orders with British Oxygen Company for the following equipment for the reasons given.

1. One or two vivostats CPV 250 Mk 2, fitted with automatic auditory low level alarms (approximately £1,600).
2. One storage tank TWN 500 fitted with CSM P/TWN 500 pump and all necessary connections and flow lines (approximately £700).

Reasons

The assurancy regarding warranty are impressive and even if the CPV 250 is worthless, we stand to lose £700. This is unlikely and must be viewed against the background of British Oxygen Company's goodwill and reputation. The back up and after sales services are good, especially the idea of insurance maintenance contracts.

If we buy Spembly we have little or no after sales servicing. In the event of breakdown, they could not give us the local support required. If the 250 litre Spembly Vivostat lost its vacuum we would have lost £1,300 together with all of our stock! This is as likely to happen to a Spembly design as a B.O.C. design. Spembly have never offered us any sort of guarantee except that it would be as good as B.O.C.'s.

B.O.C. is a very large organisation and is likely to ride market

fluctuations. It has a number of technical men all able to discuss problems. Spembly is smaller although backed by Guiness Breweries, I believe. Only one man (Mr. Wild) is available to talk to and he has now been appointed European Manager with Market Development as a second brief. Despite this he maintains, I think genuinely, his interest in Biological applications especially cryosurgical instruments.

B.O.C. were willing to give us a list of customers already using CPV 250 vivostats so that we could get an independent assessment.

APPENDIX

VISIT TO AMSTERDAM NETHERLANDS RED CROSS

BLOOD TRANSFUSION CENTRE

PROLONGED PRESERVATION OF HUMAN BLOOD AT VERY LOW TEMPERATURES

Report of a visit by Dr. Ruthven Mitchell to Central Blood Transfusion Laboratory of the Netherlands Red Cross, Amsterdam, 20 - 22nd April, 1971.

Glasgow and West of Scotland
Blood Transfusion Service.

10:5:71

Introduction

Following the successful application for a development grant of £3,000 from the Scottish Home and Health Department to set up, investigate and develop a bank of frozen human red blood cells, much of the necessary equipment has been purchased. As a sequel to this and as a fact finding mission, the S.N.B.T.A. further agreed to my visiting the European Bank of Frozen Cells controlled by Dr. K.J. Krijnen, Co-Director in Amsterdam. I had previously visited the British Army Blood Supply Depot at Aldershot and the Regional Transfusion Centre at Brentwood where the first efforts have been centred in Great Britain. Each of these Centres is using the low glycerol/rapid freeze technique developed by Dr. Krijnen and his co-workers. Difficulties have arisen in U.K. with suitable containers to freeze the blood and suitable bags with which to deglycerolise the thawed product prior to use. For these and other reasons, it was imperative before proceeding, to visit Dr. Krijnen to make a contact for the supply of washing recovery equipment and to talk about common problems.

I was in Amsterdam for two full working days. On the first day Dr. John Cash, Deputy Regional Director, South-East Region was also present. On the second day my time was spent in the Cryogenics Laboratory performing tests and recovery procedures under the guidance of Mr. de Wit, the section leader.

Our first impression of the Centre was of its large spacious accommodation sited in a suburb of Amsterdam in quiet secluded grounds. The complex is much bigger than any British Centre but I was to learn later that this Centre really represents the show case of Dutch Serology and performs not only local but also an important and exclusive National Function. Much of what goes on in the complex is concerned with this latter function. It is the major National Centre for the collection, supply and distribution of

blood and blood products, antisera, diagnostic equipment, sterile transfusable fluids and plastic infusion equipment. It acts as a central reference centre for all ante-natal and blood grouping problems. Immunochemical analyses on a national scale are done using conventional separation and diagnostic methods for the whole country (haptoglobins, paraproteins, immunoglobulins). Reports for such patients are computerised and much time is spent in administering the complicated reporting system especially to doctors many miles from the Centre.

Part of the building is occupied by the Dutch Army Blood Supply personnel, who perform all of the grouping and serology on their own army blood donors.

Blood Transfusion Matters

Donor organisation is similar to our own and I was told about five or six blood collecting teams visit wherever suitable premises can be obtained. Medical Officers do not accompany the teams and venepunctures are performed by trained technicians. With in the City of Amsterdam and throughout the country a total of approximately 100,000 units of blood is collected annually. Ninety per cent of this is obtained in glass bottles and only some 10,000 units are deplasmatised to provide "fresh" plasma and concentrated red cells. To avoid viscosity problems during transfusion these units are collected in double plastic packs with an additional smaller satellite bag containing 70 ml. of physiological saline which is pressed over and on to the cells just before use. It is somewhat surprising that since the Centre is also the National Centre for manufacturing disposable plastic taking and giving equipment (including equipment sold to other European countries, it uses such a very small proportion of plastic bags. Last year for example, only 5,000 units of cryoprecipitate were made. Dr. Krijnen said that there was a small

number of haemophiliacs in Amsterdam. The blood from sessions is often not separated until the following day and the "fresh" plasma separated at low 'g' forces is pooled and kept at -30°C for batch processing intermittently when a sizeable pool has been acquired. Units of four cryoprecipitates are lyophilised and stored after conventional ethanol/CO_2 preparation. This is stored at -20°C and reconstituted by adding 60 ml water. The technicians I spoke to were a little vague about the assay values of this material. Other products prepared in the Plasma Protein Fractionation Section were Plasma Protein Solution and Dried Plasma Protein Solution. Immunoglobulins both specific and prophylactic, P.P.S.B. (Prothrombin complex) were prepared using D.E.A.E. sephadex separation in small quantities.

The processing cold area was very busy and when I was there, certainly large pools were being processed. The technician who showed me around had formerly been in charge of this, but commented on the rapid progress made in recent years. Batches of plasma were being thawed out for processing. I was told that a good deal comes from other European countries and I saw plasma from West Germany being processed. This is returned to the sender when processing is complete.

One item of interest however was that no tests for hepatitis associated antigen were performed although there was a large animal house with rodents and rabbits for toxicity and pyrogen testing on products and infusion fluids. The equipment in the Drying Plant was impressive but different from our own. Next the plant was the regular 4°C bank of donor blood units. This was not impressive and only a small number, perhaps 250-300 units of blood were on the shelves. This was mostly glass bottles but a few bags of concentrated cells were stored, ranging from one shelf.

Cryogenics Laboratory

Before visiting this section it was interesting to hear the views of Dr. Krijnen. I had gone to Holland thinking that I was going to the centre of a large European Bank of Frozen Cells. I was most surprised when he said that there were in stock about 150 units collected in the Amsterdam area and a further 150 collected from other European countries. These were all of rare groups and the donor information and inventory were computerised and updated every month. Some units of autologous blood were available. Computer print-out was distributed to other countries' national reference centres (e.g. Dr. K. Goldsmith, London). Requests for rare blood were made once or twice a month so that there was really little demand. No attempt was made to lay down only H.A.A. negative donor units, although they were of course aware of the work of Tullis. Because of the small number of units actually transfused from the European Bank, Dr. Krijnen had no retrospective information on post transfusion jaundice. Probably because of the geographical and national boundaries to be crossed, I gained the impression that there was little "feed-back" of information. When questioned about the possible use of frozen cells in the long term management of dialysis patients and those awaiting renal transplants he did not have any plans to extend his bank or to offer such a facility. Again, I got the impression that the reason for this might have been that (a) they had not suffered any set-back in a dialysis programme, (b) they did not know the true incidence of H.A.A. among the Dutch people or the units from other countries already in the frozen bank.

My fears were confirmed when I visited on the second day the Immunochemistry Laboratory to see "H.A.A. testing". There was one technician performing micro-diffusion Ouchterlony technique using commercial reagents. The only specimens he had tested were in fact from

patients who were jaundiced and where the clinician requested a test for H.A.A. in the presence of hepato-cellular dysfunction. These requests were infrequent and when I said we were testing 400-450 blood donations a day he was astonished and agreeably incredulous. In this same laboratory other immunochemical tests were done and techniques included gel diffusion, electrophoresis, immunoelectrophoresis and column chromotography.

During the first morning of the visit to the Cryogenics Laboratory, we discussed briefly the history behind the development of frozen blood in Amsterdam, the search for suitable equipment and methods to supercede the previous Huggins' Cytoglomerator Technique and Rowe flat stainless steel and "radiator" containers. The features of the Krijnen can were elaborated and interest shown in the fact that the Finnish (Spembly) type can was less elaborate in the neck closure but, as far as could be judged, equally efficient. The costs were compared and they were even more interested when one compares less than two Dutch florins per can as against their own which are some eight to ten florins each (1 Dutch florin equals 9p). They will I believe, try out some of these cans from Spembly Products (U.K. distributor). We discussed methods of sterilising the cans and the problems of loss of paint and donor identification due to steam sterilisation. When we discussed recovery of the thawed cells we were fortunate in seeing a prototype automatic cell washer developed by I.B.M. (New York) being installed and given its first trials on deglycerolisation of frozen blood. Mr. Judson, the American worker, was enthusiastic in his praise of Amsterdam where so many technical assistants were available including a fully equipped workshop and development engineer who could design and make parts as required. This system holds much promise and consists simply of a closed circular bag rotating on a turntable with automatic feed in and decant

of washing fluids. Leaky centre joints are not a problem since bags and joint are all disposable and could cost less than five dollars compared to more than double this in the Baxter Elutramatic system.

Later on the first day of the visit, I was able to spend more time with Mr. de Wit talking about problems of freezing. We discussed safety precautions, ventilation, level alarms, manual and automatic levelling up devices, storage space utilization, inventory control and freezing of small aliquots of cells and their storage in glass, metal and plastic containers. We discussed the manufacture of liquid nitrogen on the laboratory site as compared to road tanker delivery of bulk supplies. I enclose a drawing of the lay out of the cryogenics laboratory showing the main features.

I was privileged to be offered the opportunity of actually freezing down a unit of blood under the guidance of Mr. deWit. I enjoyed this very much and the following morning was able to thaw and recover the cells from this after deglycerolisation. On this second day, I discussed methods of calculating losses and yields of cells, the general efficiency of the method, the methods of transporting the recovered cells and the shelf life of recovered blood.

On this occasion I also saw an automated method for the enzyme system 2,3 DPG which is said to influence red cell efficiency. They are conducting studies on this with CPD stored blood and frozen deglycerolised blood.

Other items of interest in the cryogenics laboratory were a system for controlled automatic freezing and preservation of human lymphocytes and a system for long term storage of human tissues in liquid nitrogen. With this latter, it is noteworthy that the Dutch Centre has a fully equipped morbid anatomy section with facilities for cryostat sections, immuno-fluorescence and electromicroscopy

together with photographic developing and processing facilities.

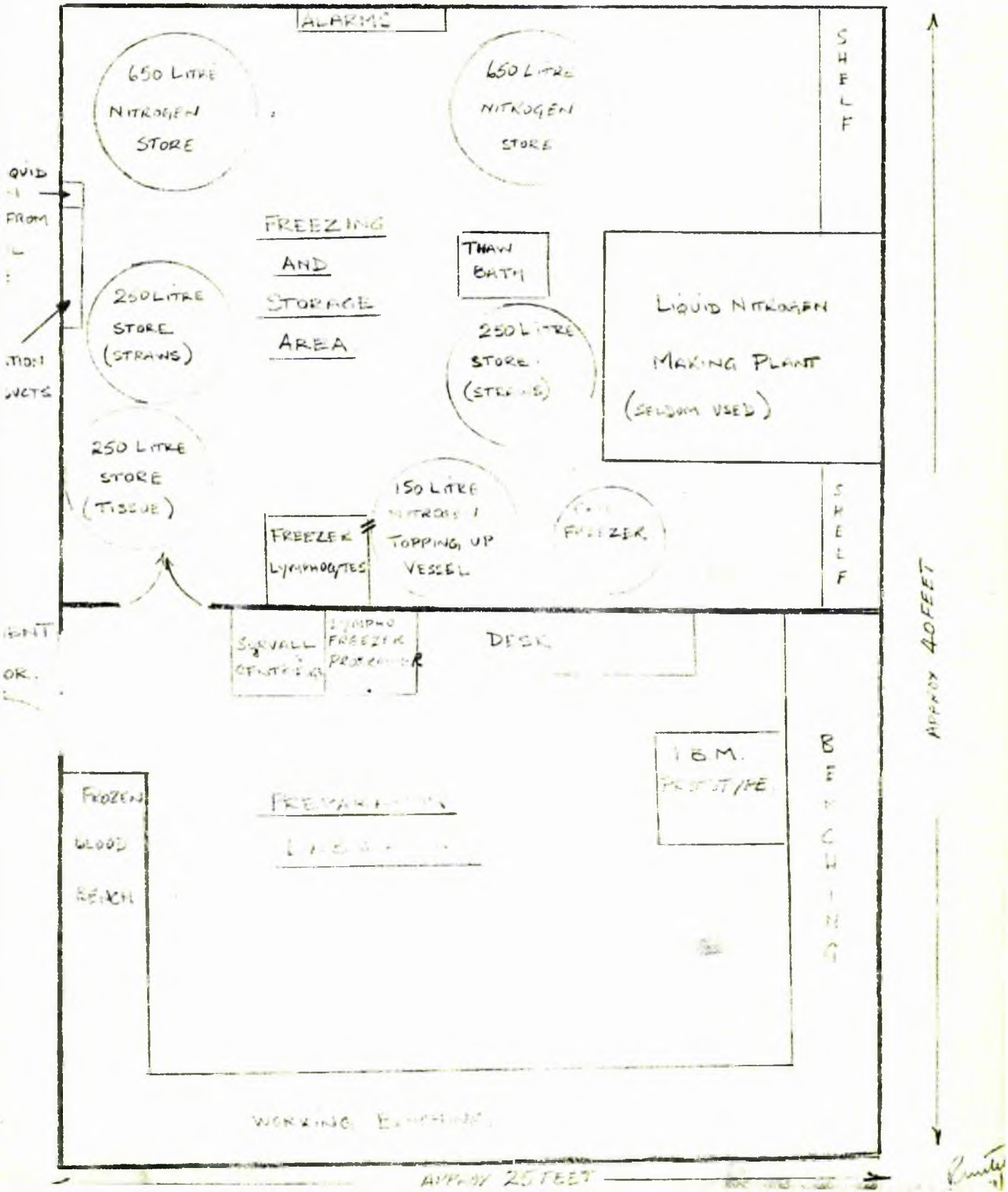
Conclusion

There can be no doubt that my visit to the Dutch Transfusion Centre in Amsterdam was immensely rewarding. I am most grateful to the Scottish National Blood Transfusion Association for granting permission for the visit. The Centre is impressive but must be viewed in the light of its age and national function it has to perform. Locally it performs as a reasonably sized Regional Transfusion Centre. The frozen blood project is limited to only rare groups and is therefore small in size. Nevertheless, considerable effort and finance have gone into developing it this far. Hepatitis and its problems are being carefully assessed at this time although we in the U.K. seem far ahead in this field. Extension of the frozen blood idea could include rare donors, autologous supplies for patients with rare antibodies and rare groups, "accredited" donations of H.A.A. negative blood for patients in dialysis units, transplant units, haematology units where leuco-antibodies must be avoided such as aplastic anaemias, chronic deficiency states associated with abnormal synthesis of abnormal haemoglobin and platelet antibodies. Sources of suitably matched cells for immunogenic stimulation of volunteers might also be considered as well as maintenance of panels of aliquots of rare cells for blood group reference work and tissue typing. In times of excess intake, suitable donations of common groups could be provided for release during periods of scarcity of supply. In the latter project it is likely that a suitable plastic bag capable of withstanding liquid nitrogen will be developed along the lines presently being evaluated in Uppsala. Automatic washing of the thawed product using the I.B.M., Electronetic (Fenwal) or other such system will greatly facilitate speed of recovery and total yield of units available at short notice. A further development will be extension of the shelf life of thawed blood. Some form of

cryoprotective agent which does not require removal after thawing would be a great advance. Less washing, faster recovery and improved shelf life will be economically favourable. At the present time, therefore, there is a need for slow but steady development to decide on these factors. Once experience has been gained it would be easier to cope with a possibly rapid increase in clinical demand. It is difficult to judge the pace of advance at this stage and the best dictum might be to hasten slowly!

CRYOGENICS LABORATORY - AMSTERDAM 1971

(NOT TO SCALE.)



APPENDIX 5

PROGRESS REPORT

Glasgow and West of Scotland
BLOOD TRANSFUSION SERVICE

TELEPHONE NOS. WISHAW 73315/8
TELEX No. 779483

REGIONAL DIRECTOR
JOHN WALLACE B.Sc., M.D., F.R.C.P.G., F.R.C.PATH.

AT LAW HOSPITAL,
CARLUKE,
LANARKSHIRE.
ML8 5ES

RM/MC

18th May, 1971

Dr. J. Wallace

Dear Dr. Wallace,

Following your letter of 22nd April and our discussions since then on Frozen Red Cells, I thought that although the various documents presented were helpful to the S.N.B.T.A. and the C.C.C., I should attempt to be more specific and perhaps outline what I think might be the future developments in a frozen blood programme. We are all aware of what has gone before, so that I shall not reiterate the reasons for using frozen blood, nor the outlets for such a product but one or two additional thoughts might be of value in helping the Association to reach a conclusion.

I think that Great Britain will progress slowly along the lines taken in the United States. Although there is one national bank based at Brentwood, I can see that logistically, further banks will develop. One national bank is of value for the extremely rare donor. Years ago 'rare' cells were kept only at a few centres. Today, with the progression of knowledge, most regional centres have fairly extensive panels of rare cells. The rarities have a habit of becoming commoner once there are sufficiently interested collectors. For the extremely rare donor such as would be kept in a national centre, the donation would be valuable because the donor may take ill and not be available; he could be on holiday or away on business when his services are required.

Computerisation of records in national banks will develop for other reasons and it is logical to imagine that frozen blood will be included in the inventory. Computers will speak to computers and rapid searching of available national and international stores will be done in a matter of milli-seconds. This is how I see the development of the national bank.

In addition however, regional banks will develop. At present in the U.K. we are seeing this. We are interested, Brentwood are interested, Bristol are interested. There are rumours that Sheffield, Leeds and even Newcastle might be interested. We are seeing the development of not only a regional bank but even extension to include hospitals within the region. The case of Guy's Hospital is a case in point. This is again very similar to the present practice in the United States. The reasons for this proliferation of banks in my view,

will be accessibility of the product, whether it be heterologous or autologous donation. Where tissue transplantation is performed, supplies of suitable donor units must be readily and quickly available and not geographically distant. The supply of units of fresh blood from specially chosen donors and donors who are already well documented can be supplemented by release of blood from a local bank. In fluctuating supply and demand situations frozen blood would not be of much use in helping to smooth out these occaions if the stocks are kept at a distance from where they can be of most value. The clinical pressures are already mounting. We ourselves, have been approached by the Dialysis Unit at the Western Infirmary. Edinburgh are obviously deeply concerned and the case which they are making out is a strong one, that is that the tragic outcome of the hepatitis in Edinburgh might have been avoided by the prophylactic use of frozen cells to the propositus, during which time she was waiting for a kidney. This prophylactic use of frozen red cells as well as recommendations to extend H.A.A. testing to all donors may be part of the recommendations of the Rosenheim Committee.

I feel that central government are encouraging a home dialysis programme to try and minimize the risks in hospital. This is laudable but there are bound to be occasions when even these home dialysis patients require hospitalisation. The costs of home dialysers at the moment are not less than 3½ thousand pounds.

Although one could claim that the costs of setting up H.A.A. testing in every regional transfusion centre would not be prohibitive, I feel that other developments should not necessarily be related to this one single factor. Even if the Edinburgh Transfusion Centre were to be successful in beginning a frozen blood programme, it would take at least six months to obtain the equipment, the trained personnel and to evaluate the methods.

I hope that these remarks will be of value.

Yours sincerely,

We have now exhausted the non-recurring development grant in purchasing cryogenic equipment. Our efforts have been successful and we now have the nucleus of a cryogenic laboratory. As we have modified equipment, designed and built equipment, we have gained considerable experience. We can now easily process donations to be stored in liquid nitrogen. The following members of staff can do this - myself, Messrs. Muir, Blue and Leitch and Miss McLaren. In the recovery phase we have been equally successful, and have achieved 97% red cell recovery in the final product. Bacteriological studies on the recovered product are being conducted at the moment, and look encouraging. Biochemistry have helped with measurement of small quantities of haemoglobin in supernate fluids. It is obvious that a team of people have become involved in various ways in the project. The next step is to consolidate by (a) streamlining the technique, and (b) allocating technical manpower.

A. Streamlining

At the moment we work in the basement. This means commuting between the centrifuges upstairs and the cryogenic equipment downstairs. In the basement we lack space, especially storage space for software equipment, which is distributed throughout the building in various other resting places, e.g. cans in stores, plastic washing bags in stores, sterile cans and fluids in citrate store-room, gloves, goggles in basement cupboard, records in my office, bacteriology and S.I. laboratory.

To avoid all of these problems would mean an area specially set aside. The modified plasma store by the drying plant should provide a satisfactory site.

To allow development of this site the upstairs portion should be fitted out as a laboratory with a kitchen type hoist fitted for

transporting specimens upstairs. This laboratory need not be very elaborate, but benching and services should be available. It is unlikely that the floor could bear much weight, and therefore all heavy equipment should be downstairs. A large laminar flow cabinet in the upstairs laboratory would be valuable or else a portion of the area closed off as a sterile work area. Two additional vivostats (250 litre) should be purchased to allow about 500 donor units to be stored as -

(a) blood for dialysis units (group O).
(b) blood for bank supplementation (homologous).
(c) heart-lung surgery (homologous).
(d) research - malarial donations: H.A.A. donations.
(e) rare groups and autologous transfusions. Aliquots for boosting volunteers?

B. Staffing

The one major hold-up in the development has been the lack of staff with sufficient uninterrupted time to devote to the numerous projects. The shortage is one of time and not ideas. Each person who has worked on the project has been frustrated by lack of space and lack of uncommitted time. Despite this, much has been achieved, but it is now obvious that for continued orderly progression two full time members are needed (a) to supervise the inventory, prepare and control reagents, and (b) freeze units when they are available and recover them when required by the research programme. Already essential work has had to be curtailed and the timetable interrupted. Supervision is increasingly difficult owing to fragmentation of effort. Results are obtained in a disconnected non-continuous manner, and good order and scientific discipline are in jeopardy. Numerous fertile ideas are being tossed aside in the hope, rather than in the belief, that they can be sacrificed for the main goal, which is the smooth functioning of a frozen cell bank. In view

of the totally new concepts involved in this project, I would think that a science graduate might be enlisted to help with the investigation, as well as the help of a technologist who is adept at innovation and novelty.

APPROXIMATE ADDITIONAL COSTS 1972/73

Two TWN 250 Vivostats	-	£1,600-00
Laminar Flow Cabinet	-	1,000-00
Aluminium Cans	-	100-00
Washing bags, etc.	-	100-00
Hoover suction; electric fan for defrosting	-	100-00
Miscellaneous aluminium, tools	-	350-00
Nitrogen liquid	-	200-00
Mistral 6L centrifuge	-	1,200-00
Ventilation	-	200-00
Dielectric sealer (Radyne)	-	500-00
Science Graduate	-	1,230-00
Senior Technician	-	1,670-00

APPENDIX 6

TECHNICAL PREPARATIVE NOTES AND TECHNIQUES

BEFORE READING THESE NOTES YOU SHOULD FAMILIARISE YOURSELF WITH THE SAFETY LEAFLETS ON THE HANDLING OF CRYOGENIC LIQUIDS.

PREPARATION AND FREEZING OF BLOOD

IN LIQUID NITROGEN

WEST OF SCOTLAND BLOOD TRANSFUSION SERVICE

July 1972

Cryophylactic Agent

Glycerol	380 gms	Make up to 1 litre with pyrogen free distilled water.
Sorbitol	29 gms	
NaCl	6.3 gms	

Wash Solution

Sorbitol	175 gms	Make up to 1 litre with pyrogen free distilled water.
NaCl	8 gms	

Preparation of Cans

1. Immerse cans in a solution of RBS 25 in 60°C water, 20 gm/litre, for 30 minutes.
2. Wash with 2% acetic acid in distilled water.
3. Rinse three times in freshly prepared distilled water.
4. Cover mouth of can loosely with tinfoil.
5. Dry at 150° for 1 hour.
6. Secure tinfoil on cans.
7. Autoclave cans in wet run at 115°C for ½-hour, with biological and chemical controls.
8. Using aseptic technique close cans with sterile sub-a-seals, with phenol glycerine under flap.
9. Wire tightly around neck with 21 SWG fuse wire, and draw indicator line on sub-a-seal.
10. Place viscap stripper wire over sub-a-seal, cover with viscap.
11. Autoclave at 115°C for ½-hour in wet run, with biological and chemical controls.

Preparation of Sub-A-Seals and Rubber Covers

1. Boil in detergent solution for 30 minutes, ½ fl. oz. sterilene to 1 gallon water.
2. Rinse three times in freshly prepared distilled water.
3. Boil in freshly prepared distilled water for 40 minutes.
4. Pack in nylon bags suitable for autoclave.
5. Autoclave at 115°C for ½-hour in wet run, with biological and chemical controls.

Preparation and Freezing of Blood in Liquid Nitrogen

1. Centrifuge donation of whole blood at 2,300 r.p.m. for 20 minutes using windshield head.

2. Using the same transfer set throughout procedure, transfer plasma and buffy coat into sterile A.R.C. flask.

3. An equal volume of glycerol/sorbitol solution is added, within one minute to the concentrated red cells, which are continually mixed.

4. The glycerolised cells are then transferred to the can. The transfer and airway needles are inserted at opposite ends of the indicator line on the sub-a-seal.

5. After transfer the needles are removed and the sub-a-seal and inside of rubber cover layered with phenol glycerine.

6. The cover is then placed over the sub-a-seal and pressure exerted to ensure removal of all air.

7. Phenol glycerine is added around base of cover.
   Note: All viscaps are removed, rubber stoppers cleaned with alcohol and hibitane and layered with phenol glycerine prior to use.

8. The glycerolised cells are mixed prior to freezing.

9. The aluminium can containing the aliquots is attached to the can by means of a length of covered wire, gauge 22.

10. Both cans are placed in liquid nitrogen to within 2" of the rubber cover of the unit can, and left for 15 minutes to freeze.

Recovery of Liquid Nitrogen Stored Unit

1. The units are thawed in a converted washing machine, with water temperature at 40 - 42°C. With continual agitation this takes about 10 minutes.

2. Using aseptic technique as in freezing procedure, the contents of the can are transferred to a five line recovery pack.

   Notes: (a) All needles are inserted away from marked line.

   (b) Remove clip before inserting transfer needle into can.

   (c) Do not insert airway while there is still pressure in the can.

3. After contents have been transferred, seal the line with metal clip and cut.

4. Centrifuge pack at 4,500 r.p.m. for 5 minutes in windshield head.

5. Using one transfer line remove supernatant into a sterile M.R.C. flask.

6. Add 230 ml sorbitol wash solution through same transfer line mixing continuously.

7. Seal the line with metal clip and cut.

8. Centrifuge at 4,500 r.p.m. for 5 minutes.

9. Repeat steps 5 - 8 with two pyrogen free saline washes instead of sorbitol wash.

10. Remove supernatant into a sterile M.R.C. flask.

11. Add 75 ml of pyrogen free saline.

12. Label final product.

Preparation and Freezing of Aliquots for Liquid Nitrogen Stored Units

1. The container used for aliquots is a polypropylene microtest tube with stopper, approximate size 4.5 cm by 4 mm internal diameter.
2. The tubes are filled with glycerolised blood remaining in the pack, and stoppered.
3. They are then placed in an aluminium can, size 6 cm by 4 cm with a screw on lid. The lid has a perforation to allow access of liquid nitrogen to the tubes.
4. The aliquot can is attached to the unit can and frozen.

Recovery of Aliquots for Liquid Nitrogen Stored Units

1. Required aliquot removed from aluminium can, immediately crushed by use of forceps, and dropped into universal of sorbitol wash solution at 37°C.
2. The universal is shaken to mix cells and sorbitol then centrifuged and the supernatant removed.
3. The cells are then washed twice in normal saline.
4. A 5% suspension of cells in saline is then prepared for compatibility tests.

APPENDIX 7

PYROGENS IN GLYCEROL

Glasgow and West of Scotland
BLOOD TRANSFUSION SERVICE

TELEPHONE NOS. WISHAW 73315/8
TELEX NO. 779483

REGIONAL DIRECTOR
JOHN WALLACE B.Sc., M.D., F.R.C.P.G., F.R.C.PATH.

AT LAW HOSPITAL,
CARLUKE,
LANARKSHIRE.
ML8 5ES

RM/MC

20th September, 1974

Dr. J.K. Smith,
Chief Chemist,
Scottish National Blood Transfusion Service,
Protein Fractionation Centre,
Royal Infirmary,
EDINBURGH.
EH3 9HB

Dear Dr. Smith,

Pyrogen Testing of Cryo-protective Agent

Mr. Watt has shown me your request for pyrogen testing of the glycerol protectant for frozen cell work.

I recall our discussing this some time ago when you told me that you might become involved in preparing the fluids for Dr. Pepper and his team. I explained to you that we do not test the glycerol for pyrogen. As far as I know no other blood freezers do so in the U.K.

We have always known that the glycerol might be pyrogenic, but in the strength used this might prove to be harmful to experimental animals if given intravenously. Because of the hyperosmolarity of the glycerol this inevitably means diluting it down to an acceptable level. Since it is known that 10% glycerol is not harmful to humans when injected intravenously, Mr. Watt diluted your sample one in four. This then falls into the counter trap that if pyrogen is present in the original fluid it will obviously be diluted. Furthermore in the final red cell recovery the cells undergo a four log wash. I think you will agree that such a wash is likely to dilute out any pyrogen to homeopathic quantities.

This is the reason that we have never worried about pyrogenicity. We have tested final washes on many occasions with no untoward effect. At the meeting on Red Cells preservation, in October, I will be presenting figures for over 2,000 transfusions of frozen cells none of which have shown any pyrogenicity.

Yours sincerely,

R. Steven Mitchell, B.Sc., M.B., Ch.B., M.R.C.Path.,
Deputy Director

APPENDIX 8

EXTENSION TO RENAL DIALYSIS UNITS.

REPORT OF MEETINGS

FROZEN BLOOD - USES IN RENAL DIALYSIS

Following my meeting with Professor Arthur Kennedy at Glasgow Royal Infirmary on 5th November, 1971 these ideas were aired.

1. Professor Kennedy is interested in using frozen cells in his patients.

2. He believes that physical washing of the cells lowers the virus B below infective levels.

3. He seemed interested enough to consider asking Almeida the basis for her "great dilution" idea.

4. He felt that studies on known H.A.A. infected blood would be promising. He was surprised that no American author had yet done this.

5. Any scheme to issue a different red cell product to each of the three dialysis units in Glasgow would not achieve results for an inordinate length of time. In his own unit the incidence of H.A.A. was zero. Frozen cells could not improve on this, although they might reduce the risk as would washed cells.

6. Turning to the issue of blood for dialysis patients who might be eventual recipients of grafted kidneys, washing of cells before transfusion would also remove W.B.C.s and lessen the risk of leucoantibody production. Frozen cells would offer the same advantage, but would be more costly at the present time.

7. Autologous transfusion offered the advantage of avoidance of any risk of transmission of disease, but increased the hazard of secondary bacterial contamination due to the larger amount of washing in the batch washing of the product recovery phase, where multiple opportunities exist for microbiological contamination.

8. Frozen blood offered a better chance of a close match for each recipient, ie. more antigens in common between donor and recipient could be achieved. This would be of value in the long term patient receiving many transfusions. Professor Kennedy, however, tends to restrict transfusion except for very low haemoglobin values.

9. Professor Kennedy speaks for the other units when he declared that they would all be willing to co-operate in any frozen cell project.

10. It is this last element of "co-operation" which worries me. The fact that we must make all the proposals to my mind suggests that H.A.A. is not a problem for them, and they therefore cannot see any immediate advantage in using frozen blood as distinct from pre-tested H.A.A. accredited washed cells.

11. Professor Kennedy suggested that we formulate our proposals and present these at a meeting with the three consultants in charge of dialysis units in Glasgow.

PROPOSAL

Object

To investigate the feasibility and desirability of introducing frozen stored reconstituted red blood corpuscles in dialysis units in the West of Scotland.

Aims

Find out how many cases require transfusion each year in the three units. Is transfusion restricted at the moment equally in all three units, or disproportionately in Professor Kennedy's unit. Is there a reluctance to transfuse at the moment because of hazards of transfusion, infective and immunological. Is there a desire to improve the patient care by improving transfusion capability by reducing these hazards. Alternatively is the balance already struck, and is it adequate for the needs now and in the future. Is simple washing of accredited H.A.A. tested donations sufficient despite the lack of sensitivity in the H.A.A. tests. Is it important to try to obtain close matching of red cell antigens of donor and recipient, especially where planned procedures are envisaged, and sufficient time exists to search the frozen cell inventory. Can the Transfusion Service deliver recovered cells at all times including holidays, weekends, evenings and during the night. Does freezing and thawing alter the virus B in any way, and does this explain the efficiency of washed thawed cells in avoiding transfusion of contaminated viraemic donations. Can we use only group O cells.

Experimental Design

1. Only known H.A.A. negative group O donations would be frozen and stored. These would be assessed for all other common antigens.

2. Storage and recovery would be at R.T.C. initially during regular working hours Monday to Friday, until sufficient staff were trained and other laboratory facilities made available.

3. Upon receipt of a request for donor blood the recipient's specimen would be typed for all common antigens. Thereafter this profile would be kept for each patient for future reference.

4. After searching the inventory suitable donations would be selected to match as closely as possible.

5. These would be recovered and despatched for use all in a period of time not exceeding 12 hours.

6. Haematological assessment of the patient before and after transfusion. This would include full haematology, red cell survival using differential agglutination and or ^{51}Chromium Studies.

7. Units for transfusion would be chosen solely on their merits as regards sharing of antigens between donor and recipient. Time of storage in liquid nitrogen would not be a major deciding factor.

USE OF WASHED, THAWED RED CELLS

A meeting was held in the Department of Medicine, Glasgow Royal Infirmary on 5th November, to discuss the use of frozen stored red cells in renal dialysis units. It was agreed to propose a scheme for adoption at a further meeting on 25th November 1971.

1. It is generally believed, although not conclusively proven, that the use of washed human red blood cells recovered from liquid nitrogen storage, offers certain advantages.

The following points emerged from general discussion:

(a) These cells are quadruple washed and this ensures a diminution in the unlikely risk of transmitting the virus of serum hepatitis (Au SH). This effect is also desirable despite the use of Au SH screened donations of blood since it is accepted that some donations that are viraemic may escape detection by present methods. It is to be noted that this same effect of washing may be physically achieved by using non-frozen red cells although in a more limited degree.

(b) Washed, thawed red cells are suspended in saline and have a limited shelf life (12-24 hours). It is presumed that physical washing (X4) effectively removes a large proportion of viable leucocytes, especially lymphocytes if these were to escape mechanical damage by the freezing process. Opinion is divided on the antigenicity of such lymphocytes and although seen very sparsely in the final transfused cells it is generally agreed that the incidence of post transfusion leucoantibody production is minimized (or even removed completely) when compared with the incidence in conventionally multiply-transfused patients.

(c) Washed, thawed red cells have no plasma present so that serum group antibody production, iso-antibody and platelet components

should not complicate their use.

(d) Using donations previously frozen and stored for 3-6 months allows time for the donor to report sick if he was in a prodromal phase at the time of donation. It is realised that not all such donors would sicken and that without a long and detailed follow up of the donor, no true accreditation can be guaranteed. It is felt that too vigorous a follow up might leave the volunteer donor being doubtful about future donations.

(e) Within the region at the present time the opinion of consultant physicians in charge of renal dialysis units is to limit transfusion. There are a number of reasons for this: the dynamic state of the circulation in patients with renal disease where subnormal haemoglobin levels are compatible with patients' requirements; the risk of hepatitis and introducing of virus into dialysis units has occurred in other places; the risk of leucoantibody induction in such patients who may thereby be precluded from kidney transplantation or treatment delayed whilst a suitable donor is found. As previously stressed, there is no convincing proof that these risks would be minimized but it might be that if they are, then the restriction on the use of blood in such patients might be less rigidly applied.

(f) It is stressed that in the Western Region of Scotland at the moment there is no evidence of Au SH virus in any dialysis unit and that only some five patients exist with leucoantibodies, one of which was transfusion induced and two by attempted grafting.

(g) Recent statements have suggested that in view of the difficulties in tracing blood donor records and the exclusion of patients from dialysis units where they have recently received either

unscreened or partially screened blood, that all patients with renal disease who might conceivably be put on a dialysis or transplant programme in the future, should receive only red cells carrying the minimum of risk achievable - i.e. washed cells from liquid nitrogen storage.

(h) It is accepted that despite the withholding of donor blood there are two occasions when infusion of red cells may be required. These are the case where haemoglobin levels are so low as to interfere with normal body metabolic requirements and where acute blood loss occurs from bursting of coils or dialysers. It is noted that in the latter case, it is usually sufficient to arrest haemorrhage, replace lost volume using plasma protein solution (P.P.S.) and as a planned procedure replace red blood cells which have been adequately screened and prepared.

(i) It is accepted that there is a paucity of information concerning the survival of donor red cells in patients with renal disease. This includes normal unfrozen cells and recovered cells stored in liquid nitrogen.

(j) It is argueable that renal patients should receive only group O red cells provided a matching compatibility test is performed. This would make the logistics of freezing much easier in that only group O Rhesus Positive and group O Rhesus Negative blood need be stored. In the unlikely event of iso-antibody production to other than Rhesus antigens developing, suitably screened donor blood would be easier to find. This latter would also be covered by extended red cell antigen profiling of frozen donations. A case might be made for the use of donations from selected siblings of patients.

(x) It is accepted that to withhold therapy and to divide or restrict it in any way would be impossible because of: the small number of patients in any one year hence an inordinately long time to collect meaningful comparative data; the need to collect information e.g. at the moment the incidence of Au ^{Sa} in patients in renal units in the Western Region is zero. no experimental design can achieve better than this. Some limited information might be gained concerning leuco-antibody production using existing information as the control series and observing any change.

Proposed Scheme

1. From a date to be decided only red cells recovered from low glycerol rapid frozen donations would be used in the Western Region for the treatment of anaemia in patients in the three dialysis units at Glasgow Royal Infirmary, Glasgow Western Infirmary and Glasgow Stobhill Hospital.
2. These donations would be tested for the presence of Australia antigen and antibody and only donations shown to be negative by existing techniques would be used.
3. Only blood group O Rhesus Positive and blood group O Rhesus Negative donations would be used. Each donation would be extensively red cell antigen profiled.
4. Only donations not more than 7 days old on the day of freezing would be preserved.
5. Where possible, donations would be stored for not less than 3 months from the date of donation before use.
6. At least 24 hours notice would be given to the Regional Transfusion Centre that red cells were required for the patient. This would be done by sending a standard request accompanied by two clotted samples of venous blood from the patient for the

tests to be done and completed within a normal working day. Compatibility tests would be performed at the Regional Transfusion Centre and sufficient integral segments would be attached to each unit of red cells to allow receiving hospital haematologists to perform repeat matching tests if they considered this desirable. The opinion of consultant haematologists in the Glasgow Western Infirmary, and Stobhill General Hospital will be sought.

At a later date when sufficient experience had been gained, and technical advances made, it would be expected that less advance notice of requirements would be needed and supply and delivery outwith normal laboratory hours would be possible.

7. In the initial period, until knowledge of efficiency was obtained, not more than two units of washed red cells would be provided at any one request except by prior agreement with the Consultant in Charge of the patient.

8. All requests would be personally authorised by the Consultant in Charge of the patient.

9. The Regional Transfusion Centre would keep consultants informed of any untoward delays due to processing failure or other difficulty.

10. Final washed cells suspended in saline would be issued to the receiving hospital consultant haematologist by the Regional Transfusion Centre by the quickest available transport.

11. Washed red cells would thereafter be transfused with the minimum of delay and in any case not more than twelve hours from the time of despatch from the Regional Transfusion Centre.

12. The Regional Transfusion Centre would conduct independent observations to determine -

(a) the continued sterility of the product.

(b) the level of recovery of cells in the final product,

(c) the general quality of the product as regards its red cell haemoglobin concentration, the volume dispensed, the cytological appearances, the red cell antigenicity related to age of storage. Later developments would include white cell recovery and antigenicity studies, enzyme and metabolic studies, electron microscopy. Possible methods of assessing infectivity of red cells recovered from known Au SH positive donations and plasma would be explored.

(d) Other uses of frozen cells in clinical situations at a Regional and National level.

13. The Regional Transfusion Centre would assist consultant haematologists so far as is practicable with independent studies on the in vivo survival of washed thawed red cells, e.g. the provision of separate aliquots of cells removed aseptically from the product for radio-chromium tagging.

14. Where practicable, vigorous studies would be pursued in the three dialysis units with a view to obtaining advanced scientific knowledge of the uses of washed thawed red cells. These studies would be determined by local circumstances but might include such items as survival in vivo, plasma haptoglobins, plasma haemoglobins and other pigments.

15. All knowledge of a scientific or general nature so acquired would be shared and would form the basis of any future publication or communication.

USE OF WASHED THAWED FROZEN RED CELLS

At the meeting which took place at the Department of Medicine, Glasgow Royal Infirmary on 25th November 1971 to discuss the memorandum 'Use of Washed, Thawed Red Cells', the following additional points emerged.

Choice of Blood

It was agreed that only group O blood would be stored for use in dialysis patients. The idea of using autologous transfusion and sibling donations was considered. Both were rejected on the grounds that insufficient blood would be collected and even this would take too long a time. Renal patients were already anaemic and therefore could not be expected to offer much as autologous donors. Only small donations could be taken and there would be a major problem of developing a new storage system and pooling facilities. Siblings as donors was attractive from the point of view of leucocyte compatibility but there was no guarantee of suitable homologous ABC groups except in identical twins.

It was calculated that to provide compatible blood for every patient would require a potential panel of some 400 donors for every unit transfused if one wished to take note of major tissue histocompatibility antigens. The system of recovery of frozen cells was aimed, among others, at removing the vast majority of leucocytes and should therefore be capable of reducing the risk of iso-immunisation to a minimum. It was agreed to await studies on leucocyte recovery in the final product and to observe transfused patients for any such iso-immunisation. It was further agreed that since dialysis patients were already being kept under surveillance as to their Hepatitis Associated Antigen status and leucocyte antibody production by virology departments and the clinical immunologist at Glasgow Royal Infirmary, that at such times and to avoid unnecessary venepunctures,

specimens from patients would be submitted to transfusion laboratories for screening for iso-antibody production to red cell antigens. This was the established practice at Glasgow Royal Infirmary and Dr. Wallace agreed with Drs. Briggs and MacDougall to make the views of the group known to Drs. Hutchison and Cumming, Consultant Haematologists at Glasgow Western Infirmary and Stobhill General Hospital respectively. It was agreed that in any case every effort would be made to minimise specimen taking from patients.

An interval of not less than two months between specimens was proposed. If, in the interim period, patients required transfusion, then an opportunity would be taken to examine the transfusion matching specimens in the manner described. Any information so obtained would be exchanged between laboratories where appropriate and also be issued in the form of a report to the Physician in charge of the patient.

Recent Correspondence

The group considered recent correspondence by Jenkins et al in the British Medical Journal on the use of thawed frozen red cells. They noted the wider extension of the use in renal dialysis patients in the Brentwood (N.E. London) Region. They felt that too great an expansion too soon might result in the uncontrolled use of frozen cells and that they would prefer to be consulted about patients outwith the dialysis units. Dr. Briggs' view, agreed by the other members was that dialysis programmes should be planned, that certain patients could be excluded and that pre-dialysis transfusion requirements could be predicted. Errors were likely to be small in number.

Dr. MacDougall cited the small number of acute dialysis patients and again stressed that these would be best dealt with in generation areas of dialysis units where there was any risk of pre-dialysis

transfusions having been given using unscreened blood or blood subsequently shown to be H.A.A. positive. Dr. Wallace reminded members of the total screening programme in the Western Region and then read the letter by Jenkins et al (B.M.J. 6th November 1971, p. 360).

Requests for Thawed Frozen Cells

Dr. Davidson asked that at Glasgow Royal Infirmary specimens from patients for compatibility testing of donations prior to transfusion should be split into two. One would be retained by the Glasgow Royal Infirmary Laboratory and the other would be sent to the Regional Transfusion Centre. Dr. MacDonald agreed that they should perform repeat compatibility tests on units of blood issued from his laboratory. It was agreed that there was no major objection to this so long as no undue delay was experienced in doubly matching units of thawed cells. The Regional Transfusion Centre would send integral segments with each unit for this purpose. Dr. Wallace stated that Drs. Hutchison and Cumming had agreed to accept the Regional Transfusion Centre's matching since this was the established practice with other matching tests which were referred to Law for various purposes where their laboratories had found matching difficulties.

Professor Kennedy agreed that requests at Glasgow Royal Infirmary would be made with two carefully labelled specimens taken from the patient in the same syringe at the same time. He asked if it was advisable to have a specific request form for such requests. It was agreed that Drs. Mitchell and Davidson should consider this and agree on a suitable request form which would be distinctive and able to be kept as a permanent record of the tests. Where possible requests would be made at 24-hours notice and regular daily B.T.S. transport would be used for transmission of specimens and matched

blood between laboratories. All issues from Law would have a 12-hour shelf life although this was subject to upward review at a future time as knowledge advanced. As experience was gained, it was probable that matched thawed red cells could be made available in emergency situations, at weekends and public holidays. At the moment such requests would be dealt with individually.

Survival Studies

Drs. Davidson and Mitchell presented information gained so far in the transfusion of a patient in Glasgow Royal Infirmary with a known leucoantibody who received eight units at intervals, and a patient at Glasgow Victoria Infirmary known to have an antibody to anti-haemophilic globulin who received four units. Clinical reports had been uniformly favourable and no allergic reactions were experienced. Haemoglobin levels had responded to transfusion and no abnormal pigments were detected in post-transfusion specimens. No information was available on measured red cell (tagged Chromium) survival times but Dr. Davidson would undertake such studies in suitable cases. It was known from others however, that survival of recovered frozen cells was as good as whole blood stored at $4^{o}C$.

Follow up of Donors

Whilst every care would be taken to use accredited donations in the frozen bank, it was felt that some effort should be made to notify donors that their donation was being used in this new project and to invite them to volunteer any obvious ill health since donating. Dr. Wallace and Dr. Mitchell agreed to consider a suitable questionnaire for this purpose but also indicated that any intensive follow up of donors involving multiple examinations and interrogations would have an adverse effect on donor morale. If donations were laid down for at least three months before use then a simple request to the donor

to notify any illness developing near the time of the donating would probably suffice. Where any untoward or unexpected occurrence involving a renal dialysis recipient was encountered then, of course; intensive efforts would be made to investigate the occurrence including a reappraisal of the donor's health if appropriate.

Commencement of the Scheme

In view of the expected pressure of work in freezing a large number of donations during the Glasgow Students' Charities Campaign at the end of January, 1972, Dr. Wallace and Dr. Mitchell proposed that the scheme should be introduced in February, 1972.

Future Meetings

It was agreed to proceed with the scheme without further meeting. Additional future meetings were not discussed but all members present would maintain correspondence.

27th January, 1972

Ruthven Mitchell

Regional Transfusion Centre,

At Law Hospital,

Carluke, Lanarkshire.

APPENDIX 9

EXTENSION TO GLASGOW WESTERN INFIRMARY

EXTENSION OF FROZEN CELLS AT GLASGOW WESTERN INFIRMARY

Following correspondence between the Regional Blood Transfusion Service and renal dialysis physicians, it was proposed that consideration should be given to the possiblity of extending the use of frozen blood at Glasgow Western Infirmary. For some time it has been obvious that since planned transfusion of dialysis patients was a practical reality, there is a need to supply such a blood product for the semi-emergency coverage of renal transplantation. On 7th July 1972, Dr. Lee, Consultant Haematologist at Glasgow Western Infirmary, visited the Regional Transfusion Centre to examine the system of freezing and recovery and to prepare a schedule of possible costs expected in setting up a small bank of frozen cells at his hospital. Thereafter, Dr. Hutchison, Dr. Briggs and Professor Kennedy discussed ways and means of raising the necessary finance. Having achieved high hopes of this, Dr. Briggs convened a meeting in the Department of Haematology, Glasgow Western Infirmary on 19th October at 2.15 p.m. and invited interested parties.

Prior to the meeting an informal visit to the proposed laboratory processing area was made by Dr. Hutchison, Dr. Lee, Dr. Wallace and Dr. Mitchell to discuss certain technical matters concerned with delivery of liquid nitrogen. These are reported as an appendix to these minutes.

Present: Dr. J. Wallace
Dr. H. Hutchison
Dr. A. I. MacDougall
Dr. R. Cumming
Dr. F. Lee
Dr. J. Davidson
Dr. R. Mitchell
Dr. J. D. Briggs

Apologies for absence were received from:
Professor A.C. Kennedy,
Dr. R.M. Lindsay

Dr. Hutchison asked Dr. Wallace to outline the project up to date. The use of frozen cells for planned transfusion in the three Glasgow dialysis units was introduced in February, 1972. This had proved successful but because of geographical separation the R.T.C. could not provide cover during emergency transplant work, especially during the night and at weekends. The Western Infirmary had therefore to use conventionally washed cells to cover these periods. Although it was probable that the shelf life of prepared frozen cells could be extended beyond 12 hours it was still the U.K. practice to impose this restriction. It was therefore proposed as a direct extension of the scheme that, if finance could be made available, a small bank of 20 or so units of frozen blood would be established at the Western Infirmary. This would require special liquid nitrogen storage facilities and recovery equipment. Already, Dr. Lee and Mr. McLennan had visited R.T.C. to discuss the acquisition of such equipment and the training of staff. Dr. Mitchell had prepared a list of items and their costing to approximately £700. The Western Infirmary staff had wished to use a new 6L refrigerated M.S.E. centrifuge and this had added an additional cost. In considering the equipment it was understood that R.T.C. would assist with the supply of some "home-made" apparatus.

Dr. Briggs then spoke of the clinical requirements. Dialysis patients were only transfused for the correction of very low haemoglobin values. It was clear that the greatest need for transfusion was to cover the renal transplants surgery and these most often occurred at very awkward times outwith normal laboratory hours. There was recent evidence that the risks of transplant rejection were minimized and much reduced by the use of frozen cells. Certainly the risks of post transfusion hepatitis were very low. Previous meetings had considered the use of autologous and/or sibling

transfusion. The problem here was to collect enough blood before the patient became too ill. Storage of small individual donations of, say, 100 cc were time consuming and would not offer much immediate benefit. Not all patients had siblings who could donate. The number of transplanted cases in Glasgow was rising every year. In recent years the numbers per annum had been 5, 11, 16, 25, This year there would be approximately 35 cases and next year a projected number would be approximately 40. At the moment there are some 26 patients awaiting transplant and it was hoped that sufficient histo-compatible kidneys would become available. The waiting time was approximately 3 - 4 months. The list was updated every month and it was agreed that copies would be sent to Dr. Lee, Dr. Davidson and R.T.C.

Dr. MacDougall asked if the frozen cells had the same quality so far as oxygen carrying capacity was concerned and what if any was the limiting amount that could be transfused at any one time.

Dr. Mitchell said that the evidence in the literature showed normal oxygen carrying capacity of recovered cells and that up to 8 units of frozen cells had been transfused in one day to a dialysis patient undergoing gastrectomy in Dundee. Other cases of 2 - 4 units per day were common. Extensive bacteriological testing of the final washed cells issued was done at the moment. No organisms had been grown. In the early phase of development at the Western Infirmary, similar bacteriological testing would need to be done to be satisfied that the extension outwith R.T.C. could achieve the same quality control. In this latter, R.T.C. would give every assistance to Dr. Hutchison in testing donations.

Dr. Hutchison said that at the moment, immediately prior to transplantation, 3 - 4 units of blood were prepared for each patient. The time taken for this was some 2½ - 3 hours and this should not be extended. The amount of time to prepare a similar quantity of frozen

cells was the same. His technicians had expressed considerable interest in the new proposal but it was felt that, at least initially, a second-on-call or "back-up" technician should be called if requests came outwith normal laboratory hours. If specimens of serum could be sent regularly to the Western Infirmary from each patient awaiting transplant then matching and compatibility testing could often be started at the first message that a transplant was imminent. This required close co-operation and Dr. Lee undertook to arrange the storage of such specimens. It was agreed that Dr. Davidson and Dr. Cumming would send such specimens to Dr. Lee. Physicians agreed to send patients' specimens every 3 months for antibody screening and 2 weeks after any transfusion. Dr. Lee would keep R.T.C. informed of any patient on the transplant waiting list who had developed any iso-antibody to red cells or who might present any special problem in the supply specially phenotyped donations, e.g. R_2R_2, R_1R_1.

Dr. Wallace suggested that the frozen bank at the Western Infirmary would consist of twelve group O rhesus D positive donations and eight group O rhesus D negative donations. The actual transfer arrangements are set out in the Appendix.

Dr. MacDougall asked if, in view of the recommendations of the Rosenheim report, provision for patients outwith the Glasgow dialysis units was envisaged. He could see that some patients might first be seen at some other hospital and be transfused by conventional methods in the interim period before being seen by a consultant in renal diseases. It was agreed that the desirability of transfusing such patients with frozen cells from the initial requirement should be drawn to the attention of Consultant Physicians by the Consultant Renal Diseases Group which meets at regular intervals.

The starting date for the proposed extension was left open and would depend on the availability of financial assistance and conversion of laboratory accommodation at Glasgow Western Infirmary Haematology Department. It was hoped that a start would be made in early 1973.

Dr. Davidson asked that if it was now agreed that frozen cells had a place in the management of transplant patients, should consideration be given to the setting up of a similar small bank at Glasgow Royal Infirmary and Stobhill Hospital.

Dr. Wallace agreed that it was desirable at this stage at least to enquire into the costs involved and to prepare estimates within the next financial year. One could not predict the outcome of the Western Infirmary scheme. It was likely to succeed and might even require extension at a not too distant date.

APPENDIX - TECHNICAL

Storage Equipment

The proposed site for the main liquid nitrogen frozen cell store using a Union Carbide LR40 container is adequate with proper ventilation to atmosphere. Unauthorised access is prevented by a locked door to the storage area. In this area would be kept three small (25 litre) MSC dewar flasks fitted with a low pressure dispenser (VLPD/MSC25) capable of delivering 2 litres/minute for top-up of the LR40 at 1-2 lb/in^{2}g. British Oxygen Company supply this as a "package deal" which also includes a Ring Trolley (RTRL/MSC25), a level gauge dipstick (DL1G/MSC25) and a Transporter (TR1000) for moving the dewar to the fill point. It is understood that the University Department of Physics will be able to supply liquid nitrogen say once / week. If not, then there is an access point at Glasgow Western Infirmary where small tanker deliveries could be made by B.O.C. Cryo Speed Service vehicles. Small vessels have been chosen

since because various split levels and steps have to be negotiated between the laboratory and the street access, it would be impracticable to handle the larger 75 or 125 litre vessels (EC75, EC125) originally proposed. For this reason also the liquid nitrogen dispenser pump (CSMPB) originally recommended is not necessary.

Delivery of Cans of Liquid Nitrogen Stored Blood

Initially the R.T.C. would prepare two LR40 main storage units (supplied by Glasgow Western Infirmary) with all the necessary partitions, insert panels and extruded aluminium storage racks. These with 12 units of group O Rhesus D positive and 8 group O Rhesus D negative frozen units in situ and filled with liquid nitrogen to 2 inches below the can necks would be sent by road to Glasgow Western Infirmary. Each unit of blood would have attached its appropriate aliquots for matching purposes and would be accompanied by a note of the donation numbers and their groups. Any special blood in reserve for a particular patient would be appropriately labelled as suitable for matching for that patient. When units were removed and used the R.T.C. would be informed. They would then send a series of replacement units. Whenever possible, units would be used in strict date order. The replacement units would be sent in an identical LR40 storage vessel by road. For thawing and recovery purposes the Regional Transfusion Centre would provide all of the necessary equipment including:

(a) a converted washing machine thawer.
(b) all of the wash fluids.
(c) the special multi-tailed plastic wash bags.

Ruthven Mitchell,
Regional Blood Transfusion Ce
At Law Hospital,
Carluke, ML8 5ES
Lanarkshire.

21st November 1972

APPENDIX 10

SPECIAL MEETING AT

ARMY BLOOD SUPPLY DEPOT

3rd April

General discussion day. To be held in Nurse Tutors' Department, Cambridge Military Hospital, Aldershot.

09.30 hrs. Coffee

10.00 hrs. Morning Session. Chairman Dr. R. Mitchell (Glasgow)

10.00 - 11.00 hrs. General topic - Methods used for Post Thaw Recovery of Red Cells.

I.B.M. Machine - Dr. Bushrod (Aldershot)

Haemonetic and Elutromatic Machine - Mr. Varney (Guy's)

Each sub-topic to last about 15 mins. Then 30 mins. general discussion to include experience of other Centres.

11.00 - 12.00 hrs. General topic - Quality and Life Span of Recovered Washed Cells.

Rejuvenation - Dr. Fraser (Bristol) 15 mins.

Supernatant Hbs - Dr. Winwick (Aldershot) 15 mins.

Resuspension Fluids and ACD - Drs. Pepper and Amir (Edinburgh) 15 mins.

Discussion - 15 mins.

12.00 - 14.00 hrs. Lunch

It is hoped to have a Cash Bar and Buffet Lunch organised in the Nurses Tutors' Department.

14.00 hrs. Afternoon Session - Chairman Dr. G. Stoddard (Guy's)

14.00 - 14.45 hrs. General topic - Other Equipment.

Cans v Bags - Dr. Blagdon (Brentwood))
- Mr. Varney (Guy's)) 15 mins.
)

Racks and records - Dr. Mitchell (Glasgow))
- Dr. Pepper (Edinburgh)) 15 mins.
)

Discussion time - 15 mins.

14.45 - 15.45 hrs. General topic - Use of Frozen Cells in Hospitals

Experience in Guy's - Dr. Stoddard

Experience in N.E. Met. District - Dr. Blagdon

Experience in Glasgow - Dr. Mitchell

Experience in Bristol - Dr. Fraser

15.45 - 16.00 hrs. Coffee

16.00 - 16.45 hrs. General discussion on future policy - Dr. Jenkins (Brentwood). Includes equipment, proliferation of Frozen Cell Banks etc.

APPENDIX 11

REPORT ON 11TH INTERNATIONAL

MEETING OF THE SOCIETY FOR CRYOBIOLOGY

REPORT ON 11th INTERNATIONAL MEETING OF THE SOCIETY FOR CRYOBIOLOGY

LONDON 4 - 8th AUGUST 1974

by

Dr. RUTHVEN MITCHELL

This meeting was the first of its kind to be held in U.K. and was jointly organised by the British Society for Low Temperature Biology and the International Society for Cryobiology, whose previous meetings had all been held in U.S.A. The venture is seen as a means of extending the interest in cryobiology of the American Society, in Europe. To this aim there was a general discussion on the evening of 6th August at which interested individuals could speak. The matter had previously been raised at the last British A.G.M. and, as expected, there was some heated argument. The American Society acknowledged that it had not been truly international and indeed had excluded Europeans. The British Society had last year extended its membership to include Europeans, although meetings were more informal and held more frequently. (4/year). Members were anxious to cooperate with Americans, but not always on their terms. It was, for example, apparent that the next international meeting was already scheduled for U.S.A. in 1977. Those present agreed this would not preclude a European meeting in any other year and, of course, European and American groups could continue to exist in parallel, holding their own local and national meetings. For example, the British Society were jointly sponsoring a forthcoming meeting at the Netherlands Red Cross Blood Transfusio Centre, Amsterdam.

About 300 delegates attended this present meeting, including a number of British Blood Transfusion Centre staff with an interest in the cryogenic applications to Blood Transfusion practice. From Scottish N.B.T.S. were myself, Dr. Cook from Inverness and Dr. Pepper from Edinburgh. Dr. Pepper had just returned from a visit to U.S.A. where he had seen many of the laboratories and staff who were presenting papers. His report of this visit will therefore be of considerable importance.

The sessions were well subscribed with good papers on the following subjects.

RED CELL PRESERVATION

The keynote address was given by Dr. H. W. Krijnen, Amsterdam, who reviewed the development of red cell preservation since the discovery of the

whether the properties ascribed to frozen red cells could be said to have been proven individually or collectively. Since H.A.A. testing had been introduced, was there now a need for frozen blood ? During discussion Dr. Rowe, New York, commented on the long term improvements which have been and still require to be made. He spoke of critical shortages in the State of Massachusetts last year and in the city of Boston in particular, where frozen red cells recovered had prevented imminent breakdown of supply. He was convinced of the value of frozen red cells and recalled that there is no single method of freeing red cells from all undesirable components except the use of frozen red cells. In addition, a frozen cell programme freed valuable plasma for component therapy. Dr. Huggins felt that to be really effective, means must be found of extending the dating period of blood. They were recovering large quantities of frozen cells daily, and with the hepatitis problem was implied a need to offer all patients screened and frozen/recovered cells. Cryobiologists were beginning to talk about what blood they would take if transfusion was required. By improving the climate of blood utilization the outdating of blood and wastage could be reduced to about 1% from 9%.

As well as the low glycerol/intermediate freezing technique, papers were presented on the role and use of Hydroxy ethyl starch as a cryoprotectant. Most of these however concerned small volume storage and little was said on the purely clinical approach of supplies for patients. Variability had been experienced due to different batches of HES as well as individual donor variability. Some measurements have been made of recovery post thaw osmotic stress stability, electrolyte changes, D.P.G. and A.T.P. levels and supernatant haemoglobins in an effort to optimize the freezing/thawing conditions, container dimensions and storage. Nothing was reported on length of storage possible and the problem of final product viscosity (14% HES) without washing was mentioned, although it was felt that it might be desirable to wash once in normal saline or normal plasma.

Papers dealing with the clinical evaluation of frozen cells revealed some useful data. One Japanese paper, although suffering from language difficulty, described (I think!) post-transfusion jaundice associated with HBAg in a dialysis patient. The original donation was negative by IEOP, but subsequently shown to be positive by RIA. This paper was extremely difficult to follow, but told of 44 dialysis patients who had received 412 units of frozen cells and gave data different from that in the official abstract. In balance it seems that the incidence in the author's hospital of post-transfusion HBAg hepatitis is of the order of 9% and this had been reduced to about 2% by use of frozen cells. Only some recent cases had been tested for HBAg, although they had used

there was no opportunity to ask questions on this paper. Many delegates expressed a desire to know the incidence of H.A.A in (a) the population and (b) patients in the same hospital who were not transfused.

Perhaps the best paper of the first session was by Dr. C. E. Huggins of the Massachusetts General Hospital, Boston. He sketched the indications for use of frozen red cells since the early experimental days of 1963-65 through the special indications of 1966-68, cardiac surgery users of 1969-70 to the special inventory control systems of 1971-74. He was sure that the use of frozen cells "significantly reduces anti-HLA and HAA". It allowed the more effective use of blood from 9% outdating to less than 1% in his hospital last year. So far 13,000 units have been transfused this year representing $\frac{1}{3}$ of his total blood bank issues. Most of this was "fresh" in that it was frozen soon after donation and used soon after recovery. Some units had been given a shelf life of up to 7 days post thaw. Since 1964 a total of some 50,000 recovered units had been transfused. This had allowed him to solve the problem of large unexpected demands for blood. To illustrate this he showed two recent "horrific" multiple injury victims who had each received in excess of 300 units of frozen cells together with large volumes of plasma and coagulation components. His work had recently undergone intense scrutiny by the United States Congress investigation team. Later, in private discussions, I was to learn that 3 investigators spent 3 weeks examining all of his records and questioning him intently in an attempt to assess the incidence of post-transfusion hepatitis. In all 50,000 frozen cells transfused they found two cases where there may have been an association. He also referred to recent studies which showed that frozen cells did not have a thrombocyte depleting effect in cardiac by-pass patients, who therefore had fewer coagulation problems.

Summing up this session, the Chairman, Dr. H. T. Meryman, recalled that three main methods of freezing were being used and each had its agonists and protagonists. All were used with clinical efficacy. Delays in development were purely mechanical and concerned engineering problems and, in the early stages of work, industry was not interested in the limited market, leaving individual workers to develop their ideas as best they could. Now, since all methods work, it was not worth comparing them too closely. The assets of long term storage were early recognised and more so now. These were long term storage of rare blood types, elimination of non-haemolytic transfusion reaction removal of microemboli, reduction of HAA infectivity, removal by destruction of leucocytes to about 1-5% of the original numbers without loss of R.B.C.'s, avoidance of thrombocytopenia in post cardiac by-pass patients and the provision of additional blood components by freeing available plasma from the increasing deplasmatization of whole blood. He outlined the main defects as being the costs, the procedural complexity of operating the system and the limited shelf life of 24 hours. In a further analysis/...

of the immunological competence of white cells to immunize dialysis recipients he recognised the fact that about half of the white cells surviving the post thaw washing would not exclude Trypan blue nor would they grow in culture. A long term survey was underway in U.S.A. in an effort to answer these questions. Results so far (no details) indicated that there was a "significant" reduction in HLA antibody when compared to recipients of whole blood. In discussion, a summary of all published data to date on the question of reduction of hepatitis, he thought that most evidence was empirical and based on clinical data. In his view the best analysis was the work of Tullis, who had described cases receiving 500 units of frozen cells without any evidence of hepatitis. Huggins' figures had not demonstrated any clearly documented case. In his own department 50,000 units of blood were frozen each year. Thirty two centres throughout American Red Cross blood banks were now equipped to freeze donations and recover these in haemonetic bowl washing centrifuges. He gave the application of frozen cells as Dialysis Patients 50% : Transfusion Reactions 3% : Transplants 7% : Other Surgical 8% : Leukaemia 8% : Anaemias 7% : Autotransfusion 8.5% : "Other Medical" reasons 6.7%. He spoke of Chaplin's conservative view of frozen cells and the following criticisms : That it was technologically not feasible, (clearly wrong); That it was too costly, especially for routine stock control (American experience clearly demonstrates that this is untrue) ; That excessive outdating will result because of 24 hour shelf life (American experience clearly shows this is untrue and indeed it provides a better usage rate) ; and That the assets of the system can be achieved by other means such as HAA testing all donations, washing, nylon filtration, dextran sedimentation, filtering etc. The American experience shows that this is untrue and all the attributes of frozen cells cannot be provided by one single method as economically.

This concluded the first session on red cell preservation and I have attempted to report the general feeling of the speakers and delegates. Clearly many things still need to be done. Much has already been achieved and we were all pleased to note that many of the questions we had pondered were answerable or steps were being taken to answer them. A number of English Blood Transfusion Centre staffs and Army personnel were present during the meeting and we were able to meet old friends and new colleagues taking their first haltering steps in cryobiology.

APPENDIX 12

SYMPOSIUM OF BLOOD

FREEZERS IN U.K. 1974

FREEZE-PRESERVATION OF RED BLOOD CELLS: PROGRESS IN THE UNITED KINGDOM

A One-Day Symposium to be held at the London Hospital, Whitechapel, London, E.1. Thursday, 3rd October 1974

MORNING SESSION

CHAIRMAN: Dr. W. d'A. Maycock

10.00 a.m.	Coffee
10.30 a.m.	Introduction by Chairman
10.40 a.m.	Indications for the use of Frozen Cells Dr. R. Mitchell Dr. Sheilagh Murray
11.15 a.m.	Freezing Techniques Dr. I. Fraser
11.40 a.m.	Recovery Techniques Dr. J. Blagdon Col. J.G. Winwick Dr. G. Stoddart
12.10 p.m.	Discussion

12.45 p.m.	Sherry Lunch

AFTERNOON SESSION

CHAIRMAN: Maj. Gen. H.C. Jeffrey

2.15 p.m.	Organisation of a Regional Frozen Cell Bank The Brentwood System - Dr. J. Blagdon The Glasgow System - Dr. R. Mitchell The Edinburgh System - Dr. D. Pepper
2.45 p.m.	Organisation of a Hospital Frozen Cell Bank Dr. G. Stoddart Dr. R. Mitchell
3.15 p.m.	Discussion
3.45 p.m.	Summing-up - Dr. W. d'A. Maycock
4.00 p.m.	Demonstrations

APPENDIX 13

METHOD FOR ACTIVATION ANALYSIS OF ALUMINIUM

Determination of aluminium in frozen red cells.

The banking of red blood cells for relatively long periods by the process of deep-freezing and storage at the temperature of liquid nitrogen is now well established. Aluminium containers are used for this purpose and one of the problems associated with the method is the possibility of contamination of the cells with aluminium from this source. Levels of aluminium normally found in red blood cell tissue are of the order of 0.1 $\mu g\ g^{-1}$ and in order to examine the problem analytically a sensitive method is obviously required. In principle, reactor neutron activation analysis can provide this sensitivity and it was therefore decided to examine appropriate cell samples by this techinque in an attempt to obtain a quantitative measure of the possible contamination problem.

When irradiated with reactor neutrons, aluminium undergoes neutron capture to give a radioisotope which can conveniently be measured by gamma-ray spectroscopy:-

$$^{27}Al \xrightarrow{(n,\gamma)} {}^{28}Al \qquad t_{\frac{1}{2}} = 2.3 \text{ minutes}, \ E\gamma = 1.78 \text{ MeV}.$$

Unfortunately, in biological samples, the method is subject to interference from other reactor neutron reactions occurring simultaneously with phosphorus and siloon also present in the sample and which give rise to the same isotope:

$$^{31}P \xrightarrow{(n,\alpha)} {}^{28}Al$$

$$^{28}Si \xrightarrow{(n,p)} {}^{28}Al$$

Clearly, the radioactive ^{28}Al produced by these reactions cannot be distingushed from that originating from the aluminium content. Since the concentration of phosphorus in red cells is normally $\sim 600\ \mu g\ g^{-1}$ this causes serious interference making invalid any attempt to determine aluminium in its presence. Fortunately, this problem can be overcome by arranging to separate the aluminium chemically from the sample before irradiation so that it can be determined free from phosphorus and silicon.

Method.

Samples were taken from the three principal stages of the preparation of red blood cells for storage and subsequent transfusion in order to determine the

aluminium concentrations. Each set of three samples originated from the same unit of domated blood so that a comparison could be made of concentrations before and after storage. . Samples were taken at the following stages:

1. Pre-freeze stage - red cells separated from whole blood, suspended in a solution containing antifreeze agents before placing in aluminium storage bottle.

2. Post-thaw stage - thawed red cell suspension taken from the aluminium bottle immediately after storage. Storage period up to six months.

3. Final product stage - red cells after thawing, washing and re-suspension in saline ready for transfusion.

Prior to analysis, the samples were further stored at $4^{\circ}C$ in plastic containers of the type commonly used in transfusion work, until required.

During the preirradiation separation of aluminium, open bench techniques were used as "clean laboratory" facilities were not available. This placed a limitation on the best blank value obtainable but in order to reduce this as much as possible precautions were taken to prevent contamination of samples and apparatus by the use of dust covers and careful cleaning procedures.

$20cm^3$ samples of red cell suspension were shaken consecutively with two $10cm^3$ aliquots of a 1% solution of acetyl acetone (BDH) in benzene to extract the aluminium. The combined benzene phases were placed in a polythene ampoule and freeze dried to remove the solvent. Before sealing up, the ampoule was filled with CO_2 to displace the air and thus reduce to a minimum problems due to the activation of argon which would otherwise have been present in significant quantities. When irradiated, argon gives rise to the isotope ^{41}Ar which has a gamma-ray with an energy of 1.29 MeV which although not causing direct interference complicates the gamma spectrum unnecessarily if not removed or reduced. The sealed ampoules were irradiated for 2 minutes in the core of the UTR 300 at East Kilbride in a neutron flux of 3×10^{12} n sec^{-1} cm^{-2}. ^{28}Al was determined by gamma spectroscopy the detector being a 3" x 3" sodium iodide crystal (Nuclear and Silica Products) coupled to a 100 channel pulse height analyser (TMC. Gamma-scope). Aluminium concentrations were calculated by comparison with standards consisting of a pre-freeze red cell suspension "spiked" with Al^{3+} and chemically separated in the same way as the samples . Reagent and container blanks were established by running the complete analysis procedure in the absence of red cell suspensions.

APPENDIX 14

ASSAY OF 2,3 D.P.G.

2,3 DPG PRINCIPLE OF ASSAY

2,3-Diphosphoglycerate (2,3-DPG) is enzymatically hydrolyzed to 3-Phosphoglycerate (3-PGA) per reaction 1.

1. $$2,3\text{-DPG} \xrightarrow[\text{(Phosphoglycolic Acid)}]{\text{2,3-DPG-Phosphatase}} \text{3-PGA} + \text{P}$$

The enzyme which catalyzes Reaction 1 is present in purified preparations of Phosphoglycerate Mutase (PGM) and is called 2,3-DPG Phosphatase. Phosphoglycolic Acid Acid (Glycolate-2-Phosphoric Acid) is needed as a stimulator of this reaction.

The resulting 3-PGA is coupled with Phosphoglycerate Kinase (PGK) and Glyceraldehyde Phosphate Dehydrogenase (GAPD) per Reactions 2 and 3.

2. $$\underset{\text{ATP}}{\text{3-PGA} +} \xrightarrow{\text{PGK}} \text{1,3-DPG} + \text{ADP}$$

3. $$\text{1,3-DPG} + \text{DPNH} \xrightarrow[(\text{High } OD_{340})]{\text{GAPD}} \underset{(\text{Low } OD340)}{\text{Glyceraldehyde-3-Phosphate} + \text{P} + \text{DPN}}$$

By measuring the decrease in optical density at 340 mu which results when the DPHN is oxidized to DPN, we have a measure of the amount of 2,3-DPG which was originally present.

(Sigma Tech. Bulletin 35-UV 12/71)

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