CRYOPRESERVATION AND LOW TEMPERATURE BIOLOGY IN BLOOD TRANSFUSION

DEVELOPMENTS IN HEMATOLOGY AND IMMUNOLOGY

Volume 24

For a list of titles published in this series see final page of this volume.

Cryopreservation and low temperature biology in blood transfusion

Proceedings of the Fourteenth International Symposium on Blood Transfusion, Groningen 1989, organised by the Red Cross Blood Bank Groningen-Drenthe

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KLUWER ACADEMIC PUBLISHERS

DORDRECHT / BOSTON / LONDON

Library of Congress Cataloging-in-Publication Data

International Symposium on Blood Transfusion (14th : 1989 : Groningen, Netherlands)
Cryopreservation and low temperature biology in blood transfusion : proceedings of the Fourteenth International Symposium on Blood Transfusion, Groningen 1989 / organised by the Red Cross Blood Bank Groningen-Drenthe ; edited by C. Th. Smit Sibinga and P.C. Das and H.T. Meryman.
p. cm. -- (Developments in hematology and immunology ; 24)
Includes index
ISBN-13:978-1-4612-8814-5 e-ISBN-13:978-1-4613-1515-5
DOI:10.1007/978-1-4613-1515-5

1. Blood--Cryopreservation--Congresses. 2. Cryopreservation of organs, tissues, etc.--Congresses. 3. Cryobiology--Congresses. I. Smit Sibinga, C. Th. II. Das, P. C. III. Meryman, Harold Thayer. IV. Stichting Rode Kruis Bloedbank Groningen/Drente. V. Title. VI. Series.
[DNLM: 1. Blood Transfusion--congresses. 2. Cryopreservation--congresses. 3. Temperature--congresses. W1 DE997VZK v. 24 / WB 356 159c 1989]
RM171.I564 1989
615'.39--dc20
DNLM/DLC
for Library of Congress 90-5078

ISBN-13:978-1-4612-8814-5

Published by Kluwer Academic Publishers,
101 Philip Drive, Norwell, MA 02061, U.S.A.

Kluwer Academic Publishers incorporates
the publishing programmes of
D. Reidel, Martinus Nijhoff, Dr W. Junk and MTP Press.

In all other countries, sold and distributed
by Kluwer Academic Publishers Group,
P.O. Box 322, 3300 AH Dordrecht, The Netherlands.

Printed on acid-free paper

Acknowledgement

This publication has been made possible through the support of Baxter, which is gratefully acknowledged.

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FOREWORD

The theme of this 14th International Symposium on Blood Transfusion is closely related to the work and scientific contributions of the Dutch cryobiology pioneer Dr. Herman W. Krijnen of the Dutch Red Cross Central Laboratory.

Dr. Krijnen was known and respected in the national and international blood transfusion community as an extremely competent scientist and a beloved and admired colleague.

Dr. Krijnen was intentionally honoured with the invitation to open this symposium on cryopreservation and low temperature biology in blood transfusion and be the guest of honour at this event. Unfortunately, Dr. Krijnen suddenly died on the first of June 1989. In honour and memory of Dr. Krijnen this symposium will therefore be dedicated to him.

Since the 10th International Symposium on Blood Transfusion in 1985 highlighted the theme of "Future developments in blood banking", major changes have occurred in the blood banking world. Most of these changes were forced upon the Blood Banks by the fear of spreading AIDS through contaminated donations. This not only led to the widespread testing of blood, but also to a more appropriate counselling of the community and the blood donors in specific. Additionally, virus inactivation techniques were introduced for those components derived from multiple donations and intended for a regular transfusion in haemophilia patients and others.

In 1987 the State Secretary of Health in the Netherlands introduced a standard registration document on factor VIII preparations. Since January 1988 the production of all factor VIII preparations in our country, including cryoprecipitate for the treatment of haemophilia, have to observe the regulations of this document. Consequently, Good Manufacturing Practice was definitely introduced in blood banking. Advances in biotechnology have caused changes in interest and policy of Blood Banks, anticipating new approaches to blood transfusion.The concept of transfusion medicine has developed into an almost mature principle with noticeable acceptance and visible implementation.

Today, this symposium will focus on the principles and techniques of preservation of blood as a transplant in its cellular and plasma components

It will also lead the way into the rapidly extending field of organ preservation and banking.

When in two years time the bell will toll for Europe 1992, blood transfusion medicine needs to have reached the state of full maturity, and policies have to be developed and set for the future. At the turn of the 80-ties, at the start of the final decade of the 20th century, more than ever blood banking needs dynamic and creative leadership supported by wisdom and confidence. I have great confidence in the science to be presented and discussed in wisdom at this symposium, which will contribute to a tradition of leadership created by you all in contributing to this series of unique Red Cross Blood Bank events.

F. Brink
Chairman of the Board of the
Red Cross Blood Bank Groningen-Drenthe

I. PRINCIPLES AND FUNDAMENTALS

METABOLISM AND PHYSIOLOGY OF CELLS AT LOW TEMPERATURES

W.J. Armitage

The strategies for preserving blood cells can be broadly devided into storage at hypothermic temperatures above 0°C and cryopreservation at –80°C or below. With tissues such as cornea there is a third option, namely that of normothermic storage by organ culture. The aim of all three approaches is to maintain viability, which may be defined as the ability of cells and tissues to perform their normal physiological functions when transfused or transplanted. Whereas organ culture aims to keep cells in as near normal a state as possible by supplying all the nutrients necessary to maintain metabolism, the use of cooling is an attempt to reduce or abolish cellular demand for energy.

Hypothermic storage

Effects of reduced temperature on cells

The effects of cooling on cellular metabolism are complex [1]. Cooling lowers the rate of chemical reactions and so has a general depressive effect on metabolism, as demonstrated by the fall in oxygen consumption that accompanies cooling [2]. Both the demand for energy, for activities such as active transport of ions and protein synthesis, and energy production, in the form of ATP synthesis, are reduced by cooling. The relation between absolute temperature, T, and the rate constant of a chemical reaction, k, is defined by the Arrhenius equation,

$$k = Ae^{-E/RT}$$

where A is a constant, E is the activation energy of the reaction and R is the gas constant [3]. Thus, for a simple reaction, an Arrhenius plot of $\log_e k$ as a function of $1/T$ yields a straight line with slope $-E/R$. In a sequence of reactions, however, each step may have a different activation energy making the overall effect of a change in temperature unpredictable. Discontinuities have frequently been reported in Arrhenius plots of complex biological reactions [e.g. 4], which emphasizes the fact that

many properties of a cell are altered by temperature, each of which may influence the overall "reaction rate" of the process being studied.

Inhibition of active and facilitated transport systems in membranes reduces the uptake of substrates by cells and upsets intracellular ionic composition resulting in the net gain of sodium and calcium and loss of potassium. The maintenance of intracellular ionic composition at normothermia is the result of a balance between passive fluxes of ions down their electrochemical gradients and the activity of pumps transporting ions against their electrochemical gradients. The activity of the Na-K pump in human erythrocytes at 5°C is only 0.25% of that at 37°C [5]. But passive fluxes tend to be less affected by temperature and may even display paradoxical behaviour such as that shown by the passive flux of potassium in human erythrocytes which falls to a minimum at 12°C and then increases at lower temperatures [6]. The net result is that the rate of ion pumping at hypothermia is unable to match the passive ion fluxes and, owing to the charge carried by intracellular proteins, there is a net gain of Na and Cl which osmotically draws in water and leads to cell swelling [7].

Hypothermic storage of blood cells

Erythrocytes can be stored at 4°C for several weeks in plasma containing an anticoagulant. They do not swell appreciably owing to the presence outside the cells of plasma proteins and citrate. But storage in an electrolyte solution without protein does lead to swelling, although this can be prevented by the addtion of mannitol [8]. The viability of erythrocytes, as determined by their survival in the circulation after transfusion, steadily declines during storage [9]. Both metabolic changes and alterations in structure and function of the plasma membrane play a role in this loss of viability [10,11]. During storage of erythrocytes, the ATP and 2,3-diphosphoglycerate (2,3-DPG) levels fall [12,13], and the loss of plasma membrane lipids is associated with reduced cell deformability [14]. The addition of adenine to the anticoagulant reduces the loss of ATP [15]. The loss of 2,3-DPG during storage increases the affinity of haemoglobin for oxygen and compromises the ability of stored erythrocytes to deliver oxygen to the tissues. Although 2,3-DPG levels are restored to a limited extend following transfusion [16], a wide range of additives has been investigated in an attempt to moderate the loss of 2,3-DPG during storage [17,18].

When platelets are stored at 4°C, they change shape from discs to spheres [19]. The disc form is a metabolically dependent state and is maintained by a circumferential bundle of microtubules that depolymerize at low temperature [20]. Reassembly of microtubules is severely compromised by only 24 hours of storage at 4°C [21]. Plasma membrane lipids are also lost during 4°C storage [22]. Storage at 4°C for only a few hours compromises platelet viability as shown by a reduction in survival following transfusion [23]. Storage at 22°C, on the other hand, also

results in morphological and metabolic changes and reduces the *in vitro* aggregation response to ADP, but subsequent *in vivo* survival following transfusion is better preserved than after storage at 4°C [24]. Even so, the duration of storage of viable platelets is stille only a few days.

Hypothermic storage of tissues

The success of a corneal graft is critically dependent on the viability of the monolayer of endothelial cells that covers the posterior surface of the cornea. This layer of cells controls corneal hydration both by acting as a passive permeability barrier to reduce the influx of solutes and water into the corneal stroma from the aqueous humour and by actively pumping HCO_3^- ions from the stroma thereby inducing an osmotic efflux of water [25,26]. Failure of endothelial function results in corneal oedema and loss of transparency. When whole eyes are placed at 4°C, the endothelial ion pump is inhibited and the cornea gradually thickens and becomes cloudy. Returning the eye to physiological temperature restores normal metabolic activity and activity of the endothelial HCO_3^- pump, and the cornea thins [27]. With increasing storage time at 4°C, however, endothelial function becomes compromised owed to insufficient supply of substrates for the albeit reduced metabolism and build up of waste products, principally lactate, in the aqueous humour. Removal of the cornea from the eye and storage in tissue culture medium containing 5% dextran partially overcomes these problems and increases the permissible period of hypothermic storage from 2 to 4 days [28,29].

Normothermic storage

Normothermic storage of tissues

One way to avoid the detrimental effects of hypothermia that limit the duration of hypothermic storage is to maintain the cells or tissue at normothermia. Cell culture techniques are well established but for technical and logistical reasons have found no place in blood banking. In the 1970s, however, organ culture techniques developed for skin were applied to the cornea [30]. Corneas maintained in tissue culture medium at 37°C were shown to be metabolically active and retained normal endothelial ultrastructure for at least 30 days [31]. Subsequent clinical results confirmed the efficacy of this technique [32].

The increase in storage time provided by this technique confers a number of significant advantages. Routine corneal grafts can be performed electively rather than as emergency procedures; a stock of corneas is always available for genuine emergencies; there is more time for tissue typing and matching where this is appropriate (immunological rejection is the main cause of corneal graft failure); the tissue is screened for microbiological contamination; and endothelial integrity is assessed [33].

Cryopreservation

The aim of cryopreservation is to cool living cells and tissues to sufficiently low temperatures to completely suppress cellular metabolism and so maintain them in a biologically stable state. Below about –130°C, no chemical reactions can take place in biological systems: cells stored at higher temperatures (e.g. –70°C) tend to be less stable, although useful long-term storage can be obtained depending on the type of cell and the conditions under which the cells are frozen. The survival of cryopreserved cells depends on their ability to cope with a range of physical, physicochemical, physiological and biochemical stresses encountered during freezing and thawing. Uncontrolled freezing is usually lethal to cells, but many cell types do survive freezing and thawing when a cryoprotectant is present in the bathing medium and when the rates of cooling and warming are controlled [34]. Different cell types vary markedly in their response to freezing and some, for example human granulocytes, have not yet been successfully cryopreserved. In contrast to isolated cells, there has been a marked lack of success in the cryopreservation of organized tissues and organs [35].

Effects of cooling and warming rates

When cell survival is plotted against cooling rate, an inverted U-shaped curve is often obtained showing that cells have an optimum cooling rate at which survival is maximal [36]. When an aqueous solution freezes, water is withdrawn from the solution to form ice thus concentrating the solutes in the liquid phase. The concentration of solutes is fixed by temperature, and as temperature falls there is a substantial rise in solute concentration: the higher the cooling rate, the more rapid is the rise in solute concentration. Mazur [37] showed that the fall in survival at cooling rates higher than the optimum was related to the inability of cells to lose water quickly enough to maintain osmotic equilibrium. Under these conditions, the cells supercool and eventually freeze internally, which is usually lethal [38]. When cells are cooled slowly, they can maintain osmotic equilibirum with their surroundings by losing water (i.e. they behave as osmometers) [39].

Thus cells cooled rapidly contain ice but are not appreciably shrunken, whereas cells cooled slowly are shrunken but do not contain ice. The mechanism of damage to slowly cooled cells has been ascribed to prolonged exposure at relatively high subzero temperatures to the adverse changes occurring in the extracellular medium, especially the increased concentrations of electrolytes [40].

Survival of frozen cells is also dependent on the warming rate. When cells are cooled at rates just higher than the optimum, they are likely to contain small amounts of intracellular ice: the survival of these cells is higher when they are warmed rapidly rather than slowly. Furthermore,

when cells are cooled at suboptimal rates, they survive better when warmed slowly rather than rapidly [41].

Cryoprotectants

Cryoprotectants are compounds that possess the remarkable property of reducing damage to cells during freezing. Although glycerol [42] and dimethyl sulphoxide (Me_2SO) [43] are perhaps the most commonly used additives for cell cryopreservation, there is a large range of chemically very diverse compounds that are able to protect cells against freezing injury [44,45]. Cryoprotectants are broadly divided on the basis of whether they permeate cells; for example, cells are usually permeable to glycerol and Me_2SO but impermeable to dextran and sucrose. As the concentration of a cryoprotectant such as glycerol is increased, cell survival rises and the optimum cooling rate becomes lower [46]. Thus cryoprotectants are effective against slow cooling injury but are ineffective against damage caused by intracellular ice formation.

But cryoprotectants can also be detrimental to cells as a result of chemical toxicity and osmotic stress. Protocols for the addition and removal of cryoprotectants can play a decisive role in determining the survival of cyropreserved cells. The osmotic stress arises because cells tend to be more permeable to water than to the cryoprotectant. When the extracellular concentration of permeating cryoprotectant is increased abruptly, osmotic equilibrium across the plasma membrane is initially restored by an efflux of water from the cell. Cryoprotectant and water then move into the cell which returns towards its normal volume. Abrupt dilution of the external concentration of cryoprotectant, on the other hand, initially causes a rapid influx of water followed by a slower return to normal volume as cryoprotectant and water leave the cell [47]. Thus rapid addition of permeating cryoprotectant causes transient cell shrinkage, while rapid dilution causes transient cell swelling. The size of these fluctuation in cell volume depends on the concentration gradient of cryoprotectant across the plasma membrane and the membrane permeability characteristics.

The extent to which a cell can tolerate changes in cell volume can be evaluated by exposing cells to a range of hypo- and hyperosmotic concentrations of a non-permeating solute [48]. Provided that the permeability characteristics of the cell have been determined, stepwise addition and dilution protocols can be calculated to maintain the cells within those tolerated volume limits [49]. Platelets are very sensitive to osmotic stress [50] and they are damaged by rapid addition and dilution of glycerol. At room temperature, platelets are approximately 4000-fold more permeable to water than to glycerol [51,52]; thus the osmotic effects of changes in extracellular glycerol concentration would be substantial. Platelet tolerance of 1 mol/l glycerol is substantially improved when the rates of addition and removal are such that platelet volume

remains within 60-130% of normal volume [53]. Platelet permeability to glycerol is also greatly influenced by temperature. Between 37°C and 25°C, glycerol permeability falls fourfold: but at 0°C, platelets are virtually impermeable to glycerol [52]. This suggests that the Arrhenius relationship does not hold for clycerol permeability over the range 0-37°C, but it also has practical implications for the use of glycerol as a cryoprotectant for platelets.

Cryopreservation of blood cells

Because of the interactions between the main cryobiological variables of cooling rate, warming rate, type and concentration of cryoprotectant, some cell types can be succesfully cryopreserved by a variety of methods. Erythrocytes can survive freezing and thawing in the absence of cryoprotectants, and survival of about 60% is achieved at an optimum cooling rate of ca 3000°C/min [54]. Clearly, such a high cooling rate is not feasible when considering the cryopreservation of the large volumes of blood required for clinical use, nor is 40% haemolysis clinically acceptable. Two methods of erythrocyte cryopreservation for clinical use were developed from early observations showing that glycerol protected erythrocytes against freezing injury and that cryopreserved erythrocytes could be safely transfused [55,56]. In the first method, erythrocytes were equilibrated with 50-50% (v/v) glycerol and cooled slowly at an uncontrolled rate to –80°C [57]. The main problems with this method are associated with the addition and, in particular, the removal of such high concentrations of glycerol without damaging the cells by osmotic stress. To overcome these problems, a second method was developed that used a lower concentration of glycerol (15-18% v/v) and a higher cooling rate (ca 90°C/min) [58]. When erythrocytes do not need to be preserved in large volumes, high survival can be obtained by using non-permeating cryoprotectants, such as dextran and sucrose, and a high cooling rate [59].

Platelet cryopreservation has been fraught with more difficulties than the cryopreservation of erythrocytes. Both glycerol and Me_2SO have been investigated as cryoprotectants for platelets with the aim of extending the very limited storage period available for these cells at 22°C [60,61]. Reported survivals of cryopreserved platelets following transfusion range from 30-80% of controls, but none of the techniques has as yet gained wide acceptance.

Cryopreservation of tissues

Two methods of corneal cryopreservation were developed in the 1960s [62,63] and some successful grafts were performed using cyropreserved tissue. The technique proved unreliable, however, and subsequent laboratory and clinical investigations showed that freezing could cause

extensive damage to the corneal endothelium [see 64 for review]. In addition to the damage caused to individual cells in a tissue by the changes in solute composition during freezing, ice formation might also physically disrupt the structural organization upon which the overall function of the tissue depends. As a result, ways to avoid ice formation during corneal cryopreservation are being sought. One way to achieve this is by vitrification, which is the transformation of a supercooled liquid into an amorphous glass in which the molecules retain the random arrangement characteristic of a liquid. This state is brought about by a substantial increase in viscosity during cooling that suppresses molecular diffusion and thus prevents ice crystallization. To achieve vitrification at practicable cooling rates, however, requires the presence of very high concentrations (>6 mol/l) of cryoprotectants. It has been shown, however, that corneas can tolerate brief exposure to a vitrifiable concentration of cryoprotectants [65].

Conclusions

Of the techniques available for the storage of cells and tissues, the simplest is that of hypothermic storage where reduced temperature is employed to lower the metabolic requirements of cells. The metabolic and physiological changes that cells undergo at reduced temperatures, however, combine to limit the period of viable storage to only a few days in the case of platelets and corneas and to a few weeks for erythrocytes. The option of normothermic storage by organ culture, which aims to maintain cellular metabolism, has been successfully exploited to extend corneal storage to 30 days. Cryopreservation is the only technique that offers the prospect of virtually indefinite storage. Erythrocytes can be successfully cryopreserved, but the cryopreservation of platelets and corneas has thus far proved too unreliable for routine clinical use.

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FREEZING AND THAWING PLASMA

R.V. McIntosh, A.J. Dickson, D. Smith, P.R. Foster

Introduction

Freezing and thawing are critical operations in industrial plasma fractionation. The aims are to preserve the clinically important activities contained in the plasma and to recover these activities as efficiently as possible for further processing. These operations are of particular importance in the preparation of Factor VIII concentrates because Factor VIII activity is a labile component of plasma and virtually all methods of production begin with the extraction of Factor VIII activity from a cryoprecipitate formed during the freeze-thaw process.

Within the overall freeze-thaw process four areas can be identified whose design and operation are influenced by the low temperature properties and behaviour of plasma. These are: freezing; cold storage; the first stage of thawing involving the tempering or conditioning of the plasma and the second stage of thawing when the plasma is melted into a liquid state.

Freezing

Solutes which crystallize

When a simple aqueous solution of a salt freezes, ice crystals form and then grow throughout the solution increasing the concentration of the solute in the remaining fluid phase. This process continues until the concentrated solute crystallizes together with any remaining water and the whole system becomes solidified. This type of freezing is refered to as "eutectic freezing" (from the Greek eu-well and taxis-arrangement) and can be represented by an equilibrium phase diagram such as that shown in Figure 1 for sodium chloride [1].

The crystaline eutectic phase forms at a definite temperature (–21°C for NaCl $2H_2O$) when the solute has reached a certain concentration (23.3% w/v or 30% w/w for NaCl) and these values are characteristic of the particular salt/solvent system.

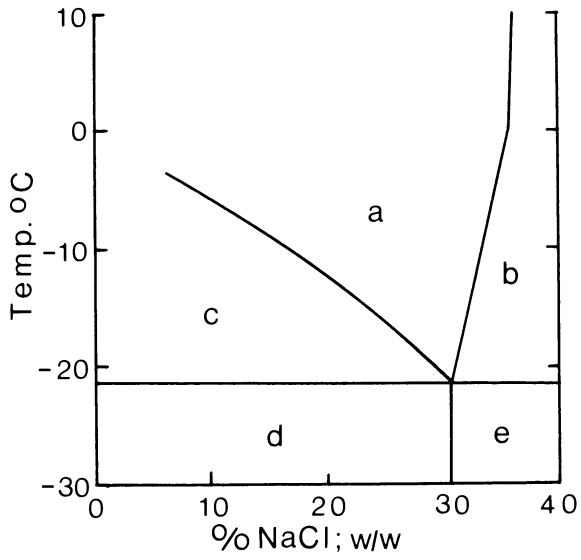

Figure 1. Equilibrium phase diagram of an aqueous solution of sodium chloride; adapted from Cammack and Adams [1]. a: Solution; b: Supersaturated solution; c: Ice + freeze concentrate; d: Ice + eutectic; e: NaCl + eutectic.

Non-crystallizing solutes

Some water soluble substances fail to crystallize during freezing. These include sugars, some synthetic polymers and proteins. A phase diagram for a model system of this type [2] is shown in Figure 2. As before the formation and growth of ice crystals is accompanied by an increase in the solute concentration of the residual liquid phase but in this case, as freezing continues, the solute fails to crystallize and instead the whole system hardens as a mixture of ice crystals in a highly concentrated viscous solution of the solute to form what is termed a "glassy" structure.

Freeze concentration

Irrespective of whether the solution freezes to a crystaline or a glassy structure a concentrated solution of solutes will be present at some time during freezing. Solute concentration increases sharply as freezing begins for example 0.15 molar NaCl is concentrated tenfold at –3°C. As well as causing changes in ionic strength, the buffering characteristics of solutions can also be affected [3]. Temperature alone alters the pKa of common buffering ions and when the buffering of a solution depends upon the molar ratio of two or more salts, such concentration effects can cause large pH shifts.

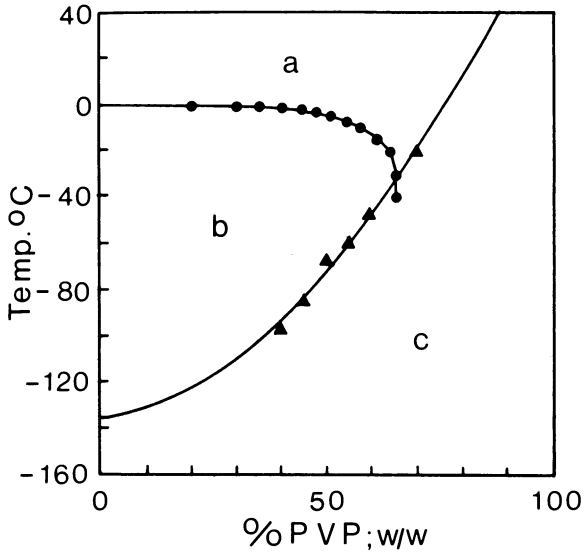

Figure 2. Phase diagram of an aqueous solution of polyvinylpyrrolidone (PVP); adapted from MacKenzie [2].
a: Solution; b: Ice + freeze concentrate; c: Glass.

In extreme cases pH shifts can occur when one salt crystallizes while the other remains in solution. The phosphate buffering systems have been studied as a good example of this effect; crystallization of the HPO_4^{-2} salt causes a decrease in pH and crystallization of the $H_2PO_4^{-1}$ form causes an increase. Similar fluctuations will occur in other systems containing for example trisodium citrate which crystallizes out at –6.9°C in simple aqueous solutions and the sodium phosphate buffer mixture which has an eutectic point at –9.9°C.

Formulation

Plasma for industrial scale fractionation is collected into a concentrated solution of additives such as citrate, phosphate, dextrose (CPD) or acid, phosphate, dextrose (ACD). These formulations were designed originally for anti-coagulation and red cell preservation in whole blood stored at +4°C and not for the cryopreservation of plasma proteins. Although formulation can be critically important in successful cryopreservation of proteins [1] there has been little or no work done on optimally formulating plasma for frozen storage. There have been some studies on collecting plasma into half-strength citrate [4] or into heparin [5,6] but these have been aimed principally at physiological improvements in Factor VIII activity. Formulation of plasma is not a topic which this paper will consider in detail.

Freezing studies on plasma

Donor plasma, unlike the systems described above is a complex aqueous mixture of salts, carbohydrate and proteins some of which (although not crystallizable) have low solubilities and are readily deposited out of solution on cooling or during freezing. It is not surprising therefore to find that there are no equilibrium phase diagrams for plasma. There have been, however, occasional studies of the freezing characteristics of plasma or related substances. Greaves [7] has compared the freezing characteristics of saline and horse serum using differential thermal analysis and the measurement of electrical resistance. Differential thermal analysis senses changes to different states by detecting as heat the energy of molecular movement which is lost as the molecules slow down and become stabilized during freezing or taken up in random molecular motion during warming. Resistance measurements exploit the property of aqueous solutions containing conducting ions that when they freeze their electrical resistance increases because of the reduced mobility of the ions. These analysis showed a clear cut step change in the sodium chloride solution during freezing and thawing corresponding to the eutectic freezing of NaCl. However, in the measurements on horse serum far less pronounced thermal changes were observed and the resistance showed a gradual change over a wide temperature range suggesting that the serum was nog completely frozen until a temperature of –70°C was reached.

MacKenzie [8,9] has presented data (Table 1) on phase transitions in human plasma detected using simultaneous differential thermal analysis and resistance measurements. These data show no simple eutectic behaviour indicating that no solid systems have been formed and different fluid states exist to very low temperatures. The continuous and gradual

Table 1. Phase transitions in donor plasma; data from MacKenzie [8,8].

Transition	**Temperature of transition (°C)**	
	Slow freezing	**Rapid freezing**
1. Glass transition (onset of motion of water molecules)	–80	–85
2. Antemelting (onset of molecular mobility of proteins)	–42 to –38	–38 to –35
3. Incipient melting (beginning of thermodynamic melting of ice)	–27	–27
4. Melting point (final melting of ice)	–0.5	–0.5

Measurement made during slow warming after slow freezing (2°C per minute) or rapid freezing (200°C per minute).

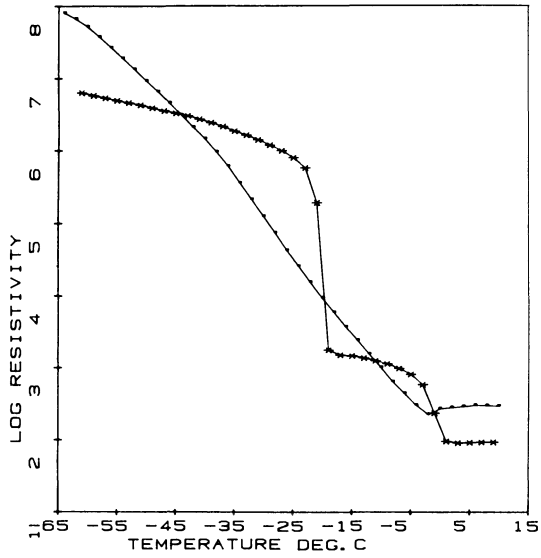

Figure 3. Resistivity of frozen plasma and normal saline. ** Normal saline; •• Plasma. Measurements made during slow thawing (at RT) after fast freezing (in solid CO_2).

nature of any phase transition in human donor plasma is confirmed in our own resistivity measurements (Figure 3) which show no evidence of any simple eutectic freezing.

Optimal freezing conditions for plasma

With so little knowledge of the events which occur during the freezing of plasma it is not surprising to find that there is no clear agreement on an optimal method for freezing plasma on a large scale. In small scale laboratory experiments Chang [10] and Over et al [11] have demonstrated the presence of sodium and protein concentration gradients on slow freezing and Over et al [11] have shown that the yield of Factor VIII in cryoprecipitate prepared from slowly frozen plasma (in –30°C stationary air) is much less than that prepared from rapidly frozen plasma (immersion in –79°C CO_2/ethanol or N_2 vapour).

Farrugia and Prowse [12] have also found in small scale experiments that slow freezing (0.33°C/min to –35°C) when compared to fast freezing (5°C/min to –60°C) has a deleterious effect on cryoprecipitate quality.

In the absence of defined freezing rate and temperature end point the trend has been to freeze plasma as rapidly as is practically possible. How-

ever, fast freezing is difficult to obtain and control at a large scale. Rowe et al [13] have noted that plasma units in the centre of a fully loaded –80°C freezer can take up to 30 h to become "frozen solid" in contrast to shorter times for units located at the bottom and top of the freezer. Smith and Evans [14] have also commented that there was no difference in Factor VIII yield from single donor packs and five litre packs, which would be expected to freeze more slowly in the same (–30°C) environment. Furthermore although freezing plasma in a –20°C or –30°C freezer has been shown to give decreased Factor VIII yields in cryoprecipitate compared with plasma frozen in much faster blast freezers [15,16], using a –40°C freezer has been reported to give yields similar to –80°C blast freezers or to freezing in liquid nitrogen [16,17].

A better approach to designing a method for the routine freezing of plasma may be to identify the principal feature of freezing which minimizes damage and determine if this can be achieved in a manner which might be easier to realize reproducibly at scale. It has been suggested [13] that prolonged freezing during the water/ice phase transition is detrimental to plasma Factor VIII activity and it has been proposed [18] that rapid ice formation helps prevent prolonged exposure to the concentration gradients which may denature proteins. Ice crystal size and distribution are dependent upon the degree to which the water can be cooled to below 0°C before crystallization begins spontaneously (nucleation). This phenomenon is called supercooling or undercooling and pronounced supercooling results in rapid ice formation throughout the solution producing a uniform dispersion of small ice crystals and a freeze concentrate of relatively uniform composition. The question then is one of how to obtain such a homogeneous frozen structure in plasma without recourse to fastfreezing. We faced a very similar problem in the pre-freezing of Factor VIII concentrate prior to freeze drying. The fastest freezing method available, which was to load the vials directly onto a –50°C cold shelf, gave a mixture of different frozen structures throughout the batch. Some vials showed an irregular heterogeneous structure containing large crystal formations others, in contrast, showed a fine crystal structure giving the freeze dried plug a uniform homogeneous appearance. We were able to obtain batches in which all the vials had the same uniform homogeneous frozen structure by using a 2-stage freezing procedure in which the product was supercooled by cooling on a –10°C shelf before freezing to –50°C [19].

The application of a similar two-stage freezing method to small aliquots (50 mls) of plasma (Figure 4) shows a sudden rise in temperature following cooling to below 0°C, indicating spontaneous ice nucleation throughout the sample. This same phenomenon should give uniform freezing throughout the entire contents of a larger sample (e.g. a plasma donation) and further work will be required to determine whether or not that is the case. The Factor VIII recoveries into cryoprecipitate prepared from the small aliquots frozen via supercooling (57%

± 17.6% n=6) were similar to those recovered from parallel samples placed directly onto a –50°C shelf (58% ± 12.3% n=6). Although these results suggest that Factor VIII activity is not adversely affected by this type of slow freezing more meaningful data on cryoprecipitation will also require larger volumes to the processed so that controlled thawing can be carried out. Although controlled thawing can be achieved with single donations [20] any benefits in terms of Factor VIII yield from homogeneous batch freezing using this method may only be evident at scale.

Cold storage

Critical temperatures in frozen plasma

Following freezing, plasma should be stored ideally at a temperature lower than that at which liquid states exist. If we consider the freezing characteristics of plasma which were presented earlier (Table 1 and Fig. 3) this temperature could be as low as –70°C or –80°C. However the amount of solution in a liquid state at temperatures close to this will be extremely small since the relationship between solid phase composition and temperature is a log/linear one (for example see Figure 3). Furthermore, in plasma the freeze concentrate at these temperatures will be very viscous and so the degree of molecular movement and denaturation will

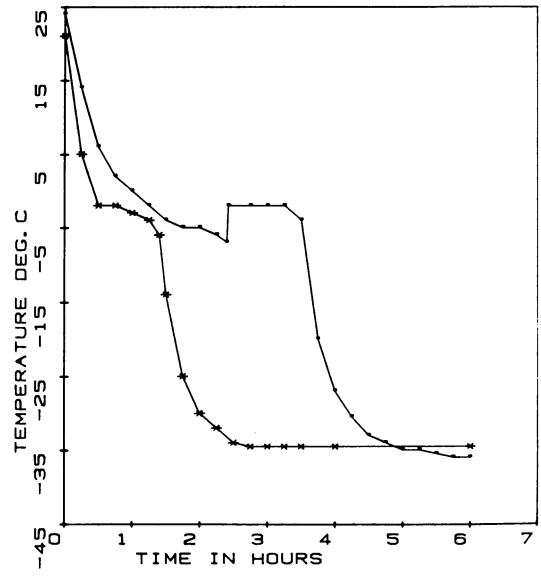

Figure 4. Freezing and supercooling in plasma. **: Plasma placed directly on –50°C cold shelf; ••: Plasma cooled to below 0°C on a –13°C shelf, then shelf temperature reduced to –50°C.

be severely limited. Nevertheless other more extensive and mobile phases exist at significantly higher temperatures such as the onset of molecular mobility of proteins (–42°C to –35°C) or the beginning of thermodynamic melting of ice (–27°C) both identified by MacKenzie [8,9] which might have more pronounced effects during long term storage.

Studies on plasma storage at different temperatures

For reasons of cost and equipment availability plasma is usually stored between –20°C and –40°C. It has been reported from small scale experiments (small aliquots or single packs of plasma) that storage at –20°C or –40°C had no effect on Factor VIII stability [22] and cryoprecipitate quality [12].

At the Protein Fractionation Centre we have studied the effects of –20°C and –40°C storage on cryoprecipitate quality at an industrial scale. Fresh frozen plasma for Factor VIII manufacture is normally stored at –40°C. However, for operational reasons, in addition to –40°C storage, some fresh frozen plasma was stored at the same time at –20°C for a limited period. A comparison of the cryoprecipitates recovered from plasma stored at these two temperatures (Table 2) showed that the mass of cryoprecipitate recovered from –20°C stored plasma was higher ($p < 0.05$) giving an overall reduction in cryoprecipitate quality (IU Factor VIII/g precipitate). This implies that –20°C storage had reduced the solubility of (e.g. partially denatured) some proteins which –40°C storage had not.

Thawing

First stage (conditioning)

Although prolonged exposure to high concentrations of solutes can be damaging to proteins it is these concentrated solutes which also have the desirable effect of forming a protein cryoprecipitate by salting out [22] or by other concentrated proteins acting as hydrophilic polymers [23]. As virtually all methods of Factor VIII production begin with the extraction of Factor VIII activity from a cryoprecipitate it is advantageous to raise the plasma temperature immediately prior to melting in a manner that ensures that cryoprecipitate formation is optimal for further processing.

Some prior warming is normally necessary to enable the plastic bag to be removed and the size of the frozen plasma blocks to be reduced by crushing for efficient metling. Forster et al [24] first noted the significance of this temperature manipulation of cryoprecipitate formation. During the development of a new crushing and thawing system it was observed that crushing plasma directly from –40°C cold storage resulted in the formation of cryoprecipitate which was more difficult to process and

Table 2. The effect of variation in plasma cold storage on Factor VIII recovery and cryoprecipitate quality.

	Cold storage °C **–40°C to –20°C** **(1 month) to –40°C**	**–40°C**
Number of batches	5	9
Batch size (l)	751 ± 80	718 ± 77
Plasma Factor VIII (IU/l)	795 ± 84	790 ± 77
Cryoprecipitate wt (g/l plasma)	11.63± 0.48	10.98± 0.57
Cryoprecipitate extract Factor VIII (IU/l plasma)	597 ± 24.9	594 ± 39.7
Cryoprecipitate quality (IU/l)	51.4 ± 2.7	54.2 ± 2.3

Results are expressed as mean ± standard deviation.

Factor VIII activity was determined by the one-stage method standardized against British Plasma Standard (87/1604) for plasma samples and British Concentrate Standard (87/1568) for cryoprecipitate samples.

Cryoprecipitate was recovered using a refrigerated multichamber centrifuge (Westfalia BKa 25).

All batches were conditioned in a Plasma Conditioning Unit at –15°C (see Table 3, column II.

which gave a lower recovery of Factor VIII activity than when the plasma was allowed to warm at +4°C for a few hours before crushing and thawing. Having established the importance of this "conditioning" effect it was considered necessary to bring it under better control by carrying out this first stage of thawing in a controlled temperature unit (Plasma Conditioning Unit) where the desired temperature could be achieved according to a selected programme, enabling the frozen plasma to be warmed from –40°C to about –10°C over about 5 hours [25].

The importance of conditioning the plasma in this manner has been demonstrated in our subsequent operational experience with this Plasma Conditioning Unit (PCU). Throughout 1985 there was a steady increase in cryoprecipitate weight while Factor VIII recoveries remained constant. This was attributed subsequently to progressive changes in heat distribution in the PCU. As Table 3 shows the cryoprecipitate weight could be reduced without affecting Factor VIII yield by lowering the conditioning end point temperature. (The cryoprecipitate weight was reduced further by returning to storage only at –40°C as shown in Table 2 and discussed above). However the deterioration in the PCU performance was followed by complete mechanical breakdown and for a period we were forced to return to conditioning plasma in a +4°C environment. A change to single donor packs (about 250 mls) from five litre pooled donations since our previous experience of conditioning in this manner meant that the plasma temperature rose more rapidly in a

Table 3. Operational data on plasma conditioning.

	Conditioning regimens			
	I	**II**	**III**	**IV**
Number of batches	18	5	36	13
Batch size (l)	750 ± 54	751 ± 80	761 ± 104	785 ± 129
Plasma Factor VIII (IU/l)	770 ± 68	789 ± 84	764 ± 130	769 ± 72
Cryoprecipitate wt. (g/l plasma)	12.21 ± 1.19	11.63 ± 0.48	10.7 ± 0.64	10.05 ± 0.34
Cryoprecipitate extract FVIII (IU/l plasma)	567 ± 47	597 ± 24.8	509 ± 42	551 ± 50.3
Cryoprecipitate quality (IU/g)	46.4 ± 4.1	51.4 ± 2.7	47.8 ± 4.6	54.4 ± 5.5

Results are expressed as mean ± standard deviation.

Factor VIII assays and cryoprecipitate weights were derived as in Table 2.

The different conditioning regimens were:

I: PCU −10°C; approx. 6 h; −8°C plasma at crushing (plasma stored at −20°C as in Tabel 2).

II: PCU −15°C; approx. 6 h; −12°C plasma at crushing (plasma stored at −20°C as in Table 2).

III: Cold room +4°C; approx. 2 h; −10 ± 2.0°C plasma at crushing (plasma stored at −40°C).

IV: PCU −16.0°C; approx. 5 h; −10.5 ± 0.5°C plasma at crushing (plasma stored at −40°C).

+4°C environment than it had previously. The results of this change where that although the cryoprecipitate weight remained low the Factor VIII yield was drastically reduced. Returning to a more controlled temperature rise in a refurbished and enlarged PCU subsequently improved the Factor VIII yield and cryoprecipitate quality (Table 3).

These results demonstrate the importance of tempering or conditioning plasma to produce an optimal cryoprecipitate. They are also consistant with the findings of Winkelman and Pinnell [26] and Farrugia et al [27] in suggesting that, within a limited range, the conditioning temperature does not influence Factor VIII yield but does affect the amount of protein which is co-precipitated with Factor VIII. Furthermore our results show that the yield of Factor VIII into the cryoprecipitate is dependent upon a controlled temperature rise during this first stage of thawing.

Second stage (ice melting)

The rate of temperature increase in the second stage of thawing (ice melting) is also critical. In the same way that rapid ice growth helps prevent protein damage on freezing so denaturation on thawing can be

Table 4. A comparison of batch and continuous thawing processes; data from Foster and Dickson [25].

	Thawing continuous	Batch
Number of batches	20	20
Batch size	965.4 ± 39.7	160.6 ± 21.1
Cryoprecipitate wt. (g/l plasma)	9.04 ± 0.64	8.49 ± 1.37
Cryoprecipitate extract FVIII (IU/l plasma)	479.3 ± 101.1	316.5 ± 60.4

Results are expressed as mean ± standard deviation.

Factor VIII activity was determined by the one-stage method standardized against British Plasma Standards (77/520) and (70/506).

In batch thawing the cryoprecipitate was recovered using a refrigerated tubular bowl centrifuge (Sharples 6-P).

In continuous thawing the cryoprecipitate was recovered using a refrigerated multichamber centrifuge (Westfalia BKa6).

minimized by rapid melting of the ice. However, rapid thawing can be difficult to control at scale resulting in local temperature overshoot causing the precipitated Factor VIII to redissolve and be lost to further processing. These problems can be overcome by using continuous thin-film thawing which is rapid and free from temperature overshoot. The development and operation of such a system which can maintain a plasma film temperature <+2°C at a thawing rate of 200 kg/h [28] has been described in detail elsewhere [24,25].

The principal benefit of this system can be seen in its comparison with batch thawing (Table 4). The continuous process showed a 51% improvement over batch thawing in Factor VIII yield into the cryoprecipitate. This difference could not be accounted for by the different amounts of Factor VIII activity remaining in the cryosupernatant, which indicated that the continuous process, as well as preventing resolution of Factor VIII during final thawing, also reduced inactivation.

Summary

In conclusion it can be said that despite the obvious importance of the freeze-thaw process in industrial scale plasma fractionation there is little published information on the nature of the freezing process in plasma or on low temperature control and manipulation during subsequent processing. Furthermore the freezing, cold-storage and thawing of plasma are all interactive such that studies on freezing for example can be compromized by less than optimal thawing or as we have shown in this report cryoprecipitation can be influenced by both storage and conditioning. However, from the available data a tentative summary of the aims of each of the four stages of the freeze-thaw process can be made:

Freezing

The aim of freezing should be to obtain a homogeneous well dispersed ice crystal structure to give a freeze concentrate of uniform composition. This may be achieved by fast freezing or by freezing more slowly but in a way which induces a significant amount of supercooling.

Cold storage

Ideally plasma should be stored at a temperature at which no fluid states exist but the use of such low temperatures may not be cost effective. For practical purposes it is necessary to adopt a temperature which avoids the more extensive and damaging of fluid states. We have observed adverse effects on cryoprecipitate following storage of plasma at –20°C and from the data of MacKenzie [8,9] an optimal storage temperature will probably lie below –27°C.

Thawing; first stage (conditioning)

To allow optimal formation of cryoprecipitate the plasma temperature needs to be raised to a suitable temperature (–15°C to –10°C) immediately prior to melting. To ensure maximum Factor VIII yield this rise should be carried out slowly (5 h) in a controlled manner.

Thawing; second stage (ice melting)

The second stage of thawing must be carried out rapidly to avoid denaturation and in a controlled way to prevent local temperature overshoot. Thin film continuous thawing as described by Foster et al [24] achieves these aims.

Acknowledgements

The authors gratefully acknowledge Paul Rogers for help with the plasma freezing experiments, Kirsty Glass for typing the manuscript and John Preece for printing the figures.

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CELL AND CELL CONSTITUENT FREEZE-DRYING: FUNDAMENTALS AND PRINCIPLES

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Introduction

The freeze-drying technique can be applied for preservation, when a compound or a product appears to be chemically, biologically or thermally unstable in solution or when different components in solution are mutually incompatible. Increasingly, guarding and control of the freeze-drying process becomes important to obtain a lyophilized product, from which the original form/structure can be recovered optimally.

Applications of the technique arose in the fourties of this century [1,2]. One out of the several reasons is sufficient to decide to freeze-dry a compound, e.g.:

- instability of the compound in aqueous environment (hydrolysis of esters and amides)
- chemical reactions between 2 components in solution
- denaturation or aggregation of biopolymers and cells resulting in loss of biological activity.

Compounds and products, which can be succesfully freeze-dried from an aqueous environment, include compounds and cells like proteins (enzymes, hormones, immunoglobulins), nucleic acids, steroids, bacteria, viruses, vaccins and lymphocytes, among others. The results is a longer chemical and micro-biological shelf-life. Of course, this yields advantages, when the compound or cell suspension is being used for therapeutic purposes, as a reference standard or just for storage during a longer period. The biological activity and the solubility of the freeze-dried product can be strongly influenced by a number of parameters. In Figure 1, a number of important parameters and their interrelationships is given. These interrelationships can be subdivided into 3 categories:

- cooling rate/product temperature / nucleation of solute/solute crystal growth / retention of matrix
- no nucleation of solute/amorphous structure / hygroscopy / collapse / activity / solubility / residual moisture / vapour pressure
- nucleation of ice/ice crystal growth/capillary diameter/sublimation rate

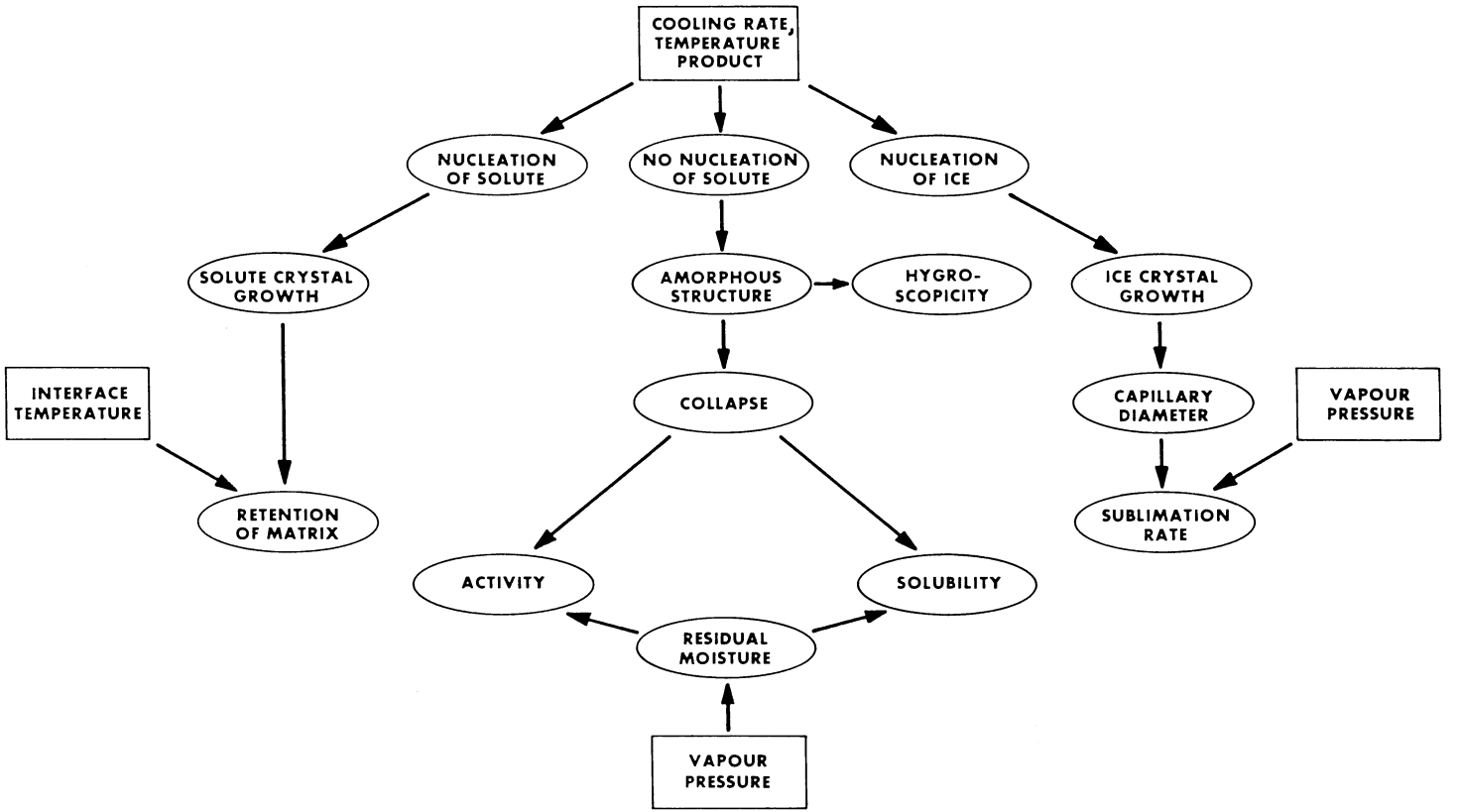

Figure 1. Interrelationships of a number of freeze-drying parameters / variables. If one parameter / varibable may exert an effect upon another, this is indicated with an arrow.

Depending upon the nature and the degree of the problems with the compound or cell suspension to be freeze-dried, attention should be paid to one or more of these parameters and is it desirable to optimize these.

Materials and methods

Freezing analyser

A freezing analyser (Edwards, Marburg, FRG) was used to determine freezing behaviour. A small sample (5.0 ml) of the dissolved product was placed in the analyser, the sample was then frozen and subsequently heated slowly under atmospheric pressure or under slightly reduced pressure to avoid condensation on the probe. During this process the temperature and electrical conductivity (AC current) were recorded simultaneously as a function of time. The calibration curve for the conductivity cell was obtained from the manufacturer (Edwards). During measurement no sublimation occurs. The analogue curves thus obtained were digitilized using a plotter (Hewlett Packard model 9872, Hewlett Packard, Palo Alto, USA) and a desktop calculator (Hewlett Packard model 9825). As the freezing analyser generates signals which are not linearly related to temperature as well as to conductivity, corrections were applied to the data read into the desktop calculator. Typically, 150-180 pairs of digitilized points were sufficient to describe the analogue curves.

Data reduction of freezing analyser curves

To extract the information contained in the analogue curves of the freezing analyser, attempts were made to describe the conductivity curves by a small set of parameters.

The following empirical model was used to calculate the parameters describing the conductivity curves:

$$-\log EC = P0 + \sum_{i=1}^{N} \{Pi,1/(1 + \exp(Pi,2(Pi,3 - T)))\} \quad (1)$$

in which:
EC = electrical conductivity measured in mS (milli Siemens); T = temperature (°C); N = number of steps; P0 = –log S at maximum temperature of experiment; Pi,1 = height of step i, = hi; Pi,2 = slope factor of step i, = Si; Pi,3 = transition temperature (Tt) of step i, = Ti.

The parameters (P) are calculated by a non-linear regression program [3,4]. A desktop calculator of 24 kbyte memory was used for relatively small datasets of about 100 points with at most two transition steps. For larger datasets or more transition steps the data were transferred to a personal computer.

The program checks for convergence by calculating the relative shift in the parameters after each iteration. If all absolute values of these relative shifts are less than $1x10^{-5}$, the program stops. If this is not the case but the relative difference in successive sums of squared residuals is less than $1x10^{-7}$, the program stops and offers the possibility to restart with different estimates. If neither of these conditions are met within 20 iterations, the program stops and returns the estimates corresponding to the smallest sum of squared residuals encountered in the 20 iterations.

The slope in each point of the curve is given by:

$$\sum_{i=1}^{N} [(Pi,1 \times Pi,2 \times (\exp(Pi,2 \times (Pi,3 - T)))) / ((1 + \exp(Pi,2 \times (Pi,3 - T)))^{2})] \quad (2)$$

This equation is valid regardless of the separation of the individual steps in the curve.

For curves with well-defined and well-separated steps this reduces to

$$\text{transition slope} = (Pi,1 \times Pi,2)/4 \quad (3)$$

Small positive or negative slope values for steps adjacent to step i yield a negligible contribution at T = Pi,3. Equation 3 can be used to obtain a starting value for Pi,2 [5].

Powder diffraction

Powder diffraction spectra were recorded (Philips pw 1050 goniometer, Eindhoven, The Netherlands) with CuK_α radiation ($\lambda = 0.154$ nm).

Moisture contents

Moisture contents were determined by injecting Karl Fisher solvent into the vial through the rubber closure. After dissolving the powder, a portion of the solution was withdrawn for coulometric determination of the residual moister content (Mitsubishi (CA-05/VA-05, Tokyo, Japan).

Scanning electron microscopy

Scanning electron microscopy was performed on a SEM S180 Cambridge instrument (Cambridge, UK) after freeze-drying.

Freeze-drying

Freeze-drying was performed on a Lyovac GT20 apparatus, (Leybold-Heraeus GmbH, Cologne, FRG), equipped with microprocessor control LPCl and vacuum lock to withdraw samples under vacuum for analysis.

Opalescence

The opalescence of reconstituted solutions was determined by turbidimetry (TRM-L, M.R. Drott K.G., Vienna, Austria) or by fluorimetry (Perkin Elmer 204, Perkin Elmer Corp. USA)

Liposomes

Phospholipon 100H (hydrogenated soybean phosphatidylcholine: PC-H) was gift of Natterman (Köln, FRG). This product contains at least 93% phosphatidylcholine, less than 2% lysophosphatidylcholine and about 2% water. The fatty acid composition is 10% $C_{16:0}$, 90% $C_{18:0}$ and traces of $C_{20:0}$. The transition temperature of PC-H in an aqueous dispersion measured by differential scanning calorimetry (Perkin Elmer DSC-2, Perkin Elmer Corp., USA) was 51°C. Dicetylphosphate (DCP) and cholesterol were supplied by Sigma Chemicals (St. Louis, MO, USA). 5,6-Carboxyfluorescein was obtained from Eastman Kodak Co. (Rochester, NY, USA). Glycerol met the requirements of the Ph. Eur., mannitol of the USP XXI. Apart from CF all chemicals were used as such. CF was purified; multilamellar vesicles were prepared with the classical film method as described previously [6]. PC-H and DCP (molar ratio 10:1) were dissolved in chloroform in a pear shaped vessel. The chloroform was evaporated in a rotary evaporator to yield a film. Traces of chloroform were removed in a vacuum desiccator for 2 h. Then glass beads were added and the film was hydrated in a 50 nmol/l CF and 10 mmol/l Tris containing aqueous solution (pH 7.4) at 70°C by hand shaking. The phospholipid concentration after hydration was around 30 mmol/l. Lipid phosphorus was determined according to the procedure of Fiske and Subbarow [7]. The principle and details of the determination of the CF retention were described elsewhere [8]. The fluorescence signal (Perkin Elmer fluorescence spectrophotometer 204, Hitachi Ltd. Tokyo, Japan) of a diluted liposome dispersion before and after destruction of the liposomes by Triton X-100 (1%) (BDH Chemicals Ltd, Poole, UK) was detected at 515 nm. The excitation wavelength was 490 nm. For complete release of CF from the liposomes the Triton X-100 containing dispersions were heated for 1 h at 100°C in sealed vials. Particle diameters were measured by dynamic light scattering (Nanosizer, Coulter Electronics Ltd, Luton, UK).

Glass vials containing 0.6 ml of the dispersion were placed in the cryostat (Julabo F40/HC, Seelbach, FRG). After freezing under the selected conditions the vials were taken out of the cryostat and thawed at 4°C. For measuring the conductivity as a function of the temperature a freezing analyser (Edwards, Marburg, FRG) was used. A 5 ml sample of the solution or dispersion was transferred to the freezing analyser. The sample was frozen down to –60°C at a rate of 7°C/min and subsequently slowly heated to +4°C or +12°C. During this heating process the temperature and electrical conductivity (AC) were recorded simultaneously as a function of time. The calibration curve for the conductivity cell was

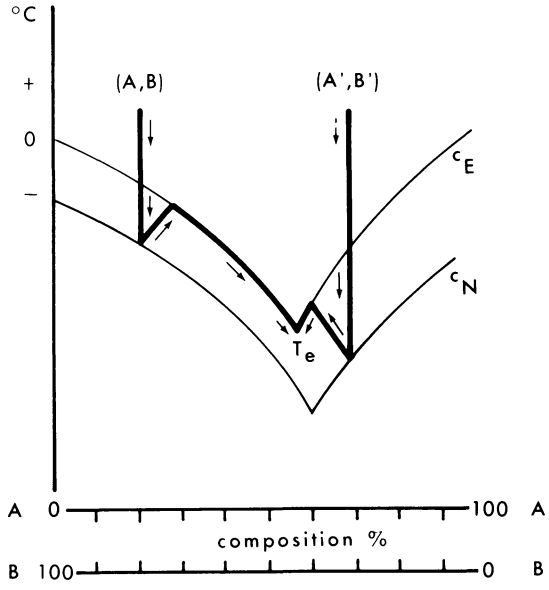

Figure 2. Phase diagram of a binary system (A,B). The eutectic point is denoted by T_e. Equilibrium melting curve and nucleation curve are denoted by c_E and c_N respectively. The bold line shows, how the composition (A,B) or (A′,B′) at time t=0 changes during freezing: the intersection of this bold line with the nucleation curve yields the temperature, at which the first crystals of compound A or B are formed.
During this process heat of crystallization is released and – as a result – the bold line rises up to the equilibrium melting curve. On further withdrawal of heat (cooling), the composition (A,B) or (A′,B′) changes conform the curve c_E in the direction of the arrow to the eutectic point T_e. The binary system can be a protein or hormone (A) solution in water (B).

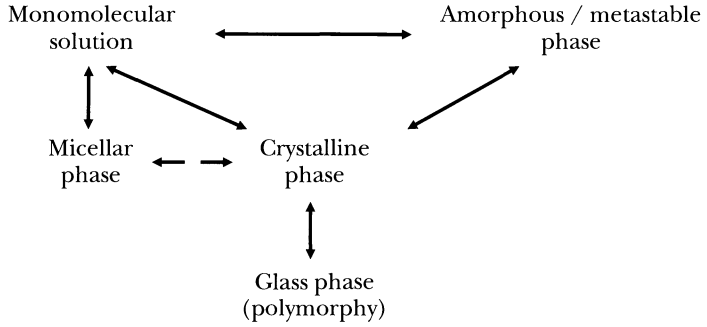

Figure 3. Phase changes, which can be encountered during freezing and subsequent freeze-drying.

obtained from the manufacturer (Edwards, Marburg, FRG). For analysis of the experimental data, the analog curves were digitized with a Hewlett Packard 9872 plotter and a Hewlett Packard 9825 desktop calculator [5,6].

Purification and chromatography of tRNA

Specific tRNAs were extracted from E. coli bacteria and subsequently purified and characterized [9]. The purification process is based upon multiple preparative liquid chromatography. The individual chromatographic runs have been interchanged with freeze-drying runs to concentrate and preserve the tRNA during ongoing purification.

Chromatographic detection and purity determination of individual tRNAs was performed using a specific enzyme radio-isotope assay [9].

^{31}P NMR spectra of tRNA

^{31}P NMR spectra were recorded on a Varian XL-100 spectrometer, operating in the Fourier transform mode at 40,5 MHz. Heteronuclear proton noise decoupling was used to remove the J coupling induced by the ribose protons. A pulse width of 20 us was employed, corresponding to a flip angle of 45°. Accumulation proceeded during 16-20 h with a spectral width of 2000 Hz and an acquisition time of 1 s; no pulse delay was used. Usually, a sensitivity enhancement was applied, yielding a line broadening of 0,6 Hz (Varian, Palo Alto, USA).

Reported chemical shifts are given relative to 20% H_3PO_4 as an - external reference with downfield shifts defined as positive. tRNA samples were dissolved in 0,2 ml of D_2O buffer, containing 30 mM cacodylate, 80 mM NaCl, 1 mM EDTA, and 10 mM $MgCl_2$, at pH 7,0. The D_2O solvent was used as an internal field frequency look.

Phase diagram

Prior to freeze-drying, knowledge of the phase diagram of the system is of ultimate importance in order to:
- know the position of the eutectic point or eutectic traject
- recognize the presence of polymorphism and metastable phases

Knowledge of the eutectic point T_e c.q. traject is essential, because during sublimation the temperature of the solid mass should be 5-7°C below T_e. Knowledge of polymorphic behaviour is essential, because the properties of the freeze-dried product – e.g. the rate of solubilization and the clarity/opalescence after reconstitution – can depend to a large degree upon the morphology obtained.

Figure 2 yields a phase diagram of a simple, binary system: the thick line has been drawn to illustrate how the composition of the binary system changes during freezing, before finally T_e is reached. In Figure 3 a survey of a number of phase changes is given, which can be encountered during

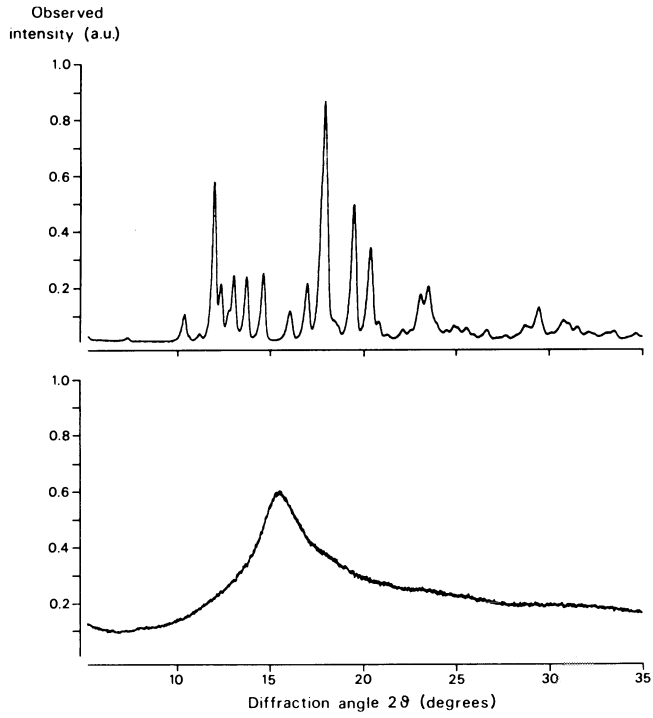

Figure 4. X-ray diffraction spectra recorded on crystalline (upper spectrum) and amorphous (lower spectrum) corticosteroid.

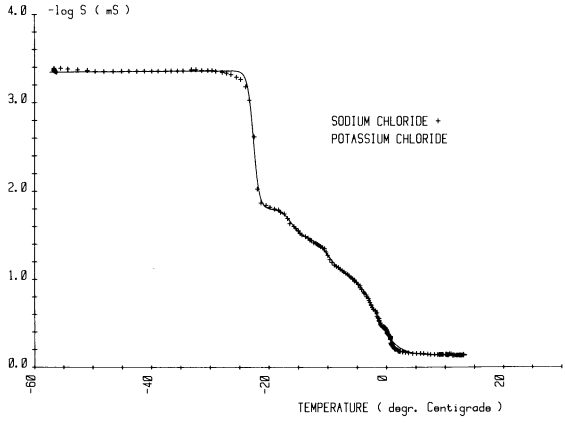

Figure 5. Digitized form (+++) of the conductivity curves for NaCl-KCl-H_2O, together with the respective simulated curve (soldid line).

freezing and subsequent freeze-drying. Understanding of phase transitions is necessary for the design of freeze-drying processes.

In order to construct phase diagrams, a number of techniques can be used, among which:
- Differential Scanning Calorimetry (DSC)
- Differential Thermal Analysis (DTA)
- X-ray differentiation
- Electron microscopy
- Electrical conductivity measurements

Examples of two techniques will be discussed below, i.e. X-ray differentiation and electrical conductivity.

X-ray diffraction in relation to phase transitions

Phase transitions can play a crucial role in the manufacture of lyophilized products. These phase transitions and large structural changes can occur at narrow temperature intervals (e.g. within 3°C) upon freezing [10]; this underlines the significance of knowledge of the phase diagram and/or temperature control of the process. These structural changes can drastically affect the dissolution properties of the freeze-dried product.

In some cases this results in an unacceptable reconstitution of the product reflected by long dissolution times or by permanent opalescence of the solution formed upon addition of solvent to the product.

As an example, different antibiotics can be mentioned [11]. Some posses an amorphous structure after freezing, associated with a bad reconstitution and hygroscopical nature. By means of a short heat treatment of the frozen mass (prior to sublimation), these antibiotics could still be obtained in crystalline form. This resulted in an improved reconstitution and in less hygroscopicity than in the case of the amorphous modification.

Crystallinity or amorphous state can be measured by recording X-ray diffraction spectra. In Figure 4 X-ray diffraction spectra of a crystalline and an amorphous corticosteroid after freeze-drying are given. The spectrum of the crystalline material is characterized by a large number of discrete Bragg reflections, whereas the spectrum of the amorphous material shows a single, broad scattering peak [5].

Electrical conductivity determination to establish the phase diagram

The information contained in the electrical conductivity curves measured as a function of temperature can be represented by a small set of parameters. This is achieved by approximating the electrical conductivity curve as a number of consecutive steps, using a suitable empirical model. The three parameters describing each step are: transition temperature, slope factor and step height. Transition temperatures

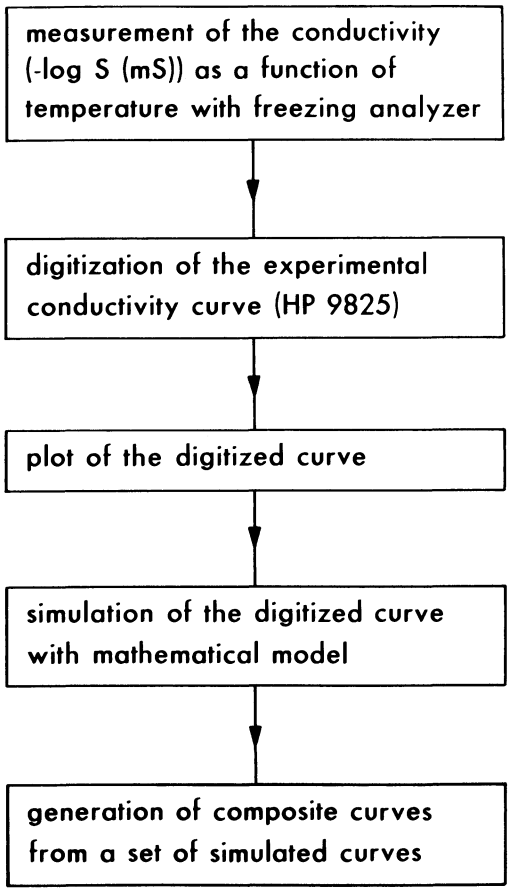

Figure 6. Flow sheet of eutectic pointdetermination by means of the freezing analyser.

Table 1. Calculated transition témperature (°C for the binary systems NaCl-H_2O and KCl-H_2O as well as for the ternary system NaCl-KCl-H_2O in comparison with their eutectic termperatures reported in the literature.Calculated transition slopes ($°C^{-1}$) are also given.

	Calculated transition temperature Pi,3 (°C)	**Eutectic slope at Pi,3 temperature***	
		(°C)	($°C^{-1}$)
NaCl-H_2O	–21.3	–21.8;–21.1	–0.82
KCL-H_2O	– 9.8	–11.1;–10.7	–4.67
NaCl-KCl-H_2O	–22.6	–23.7	–0.78

*Reported in the literature [12,13].

measured and calculated in this way are in good agreement with the eutectic temperatures reported in the literature. The major advantage of these calculations is, that complete conductivity-temperature curves are represented as a small set of relevant parameters, which can be subsequently employed in actual freeze-drying [5]. To establish the validity of the empirical model of equation 1, the transition temperatures $P_{i,3}$ of the binary systems NaCl-H_2O and KCl-H_2O as well as of the ternary system NaCl-KCl-H_2O as model compounds were calculated by non-linear regression and compared with the values of their eutectic temperatures reported in the literature.

The curves for NaCl and KCl have been recorded up to 12°C and 6°C so as to include the melting of ice crystals at 0°C, which is visible as a separate transition. Thus, both curves have been simulated using two steps in the model of equation 1. Both curves show one major transition, which is due to melting of crystallized salt.

Simulation of the NaCl-H_2O curve yielded a transition temperature $P_{2,3}$ equal to –21.3°C, which is in excellent agreement with the eutectic temperatures of –21.8°C and –21.1°C reported in the literature [12,13]. The transition temperature $P_{1,3}$ corresponds to the melting of ice crystals and will not be further discussed here. Simulation of the KCL-H_2O curve yielded a transition temperature $P_{2,3}$ equal to –9.8°C. The latter value also corresponds well with the reported values of the eutectic temperature of KCL-H_2O, i.e. –11.1°C and –10.7°C [12,13].

In Figure 5 the digitized points of the conductivity curve for the NaCl-KCl-H_2O ternary system (30 mM NaCl; 120 mM KCl) are represented by (+++). The curve has been simulated using four steps in the model of equation 1 (solid line). Simulation of the curve yielded a lowest transition temperature $P_{4,3}$ equal to –22.6°C. This value corresponds well with the reported eutectic temperature of –23.7°C [13].

In conclusion, the data analysis of equation 1 yields transition temperatures $P_{i,3}$ in close agreement with the respective eutectic temperatures reported in the literature (see also Figure 6). In Table 1, the calculated and literature values of the transition temperatures are given, together with the calculated values of the transition slopes. In a comparable way, eutectic temperatures of solutions of hormones and proteins have been measured and calculated [14,15].

Computer control and vacuum-lock sampling

The high requirements, which should be obeyed, make control and guarding of the freeze-drying process necessary; this holds particularly for biological products with their specific physico-chemical properties. To ensure an optimal process, modern freeze-drying instrumentation is supplemented with microprocessor or computer control of the process and with vacuum-lock sampling.

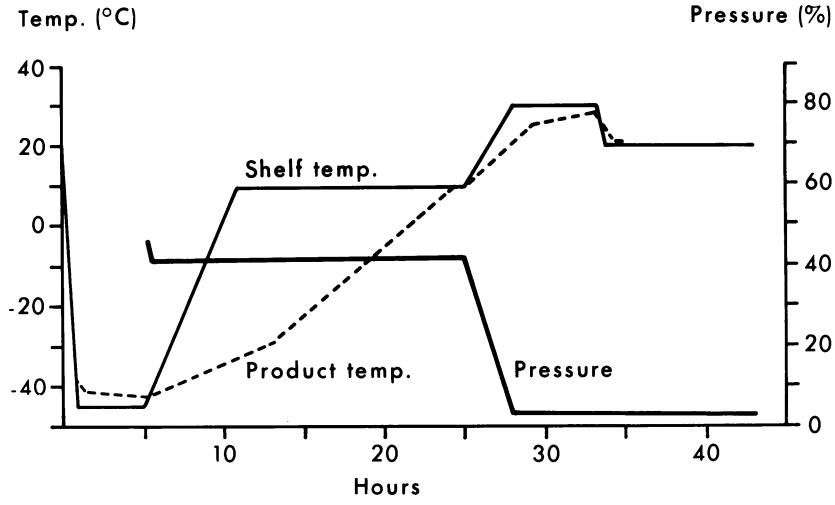

Figure 7. Temperature and pressure profile during a normal freeze-drying cycle, i.e. freezing, sublimation (primary drying) and after-drying (secondary drying). The course of shelf and product temperature is separately indicated. The onset of after-drying is visible as the second increase of temperature.

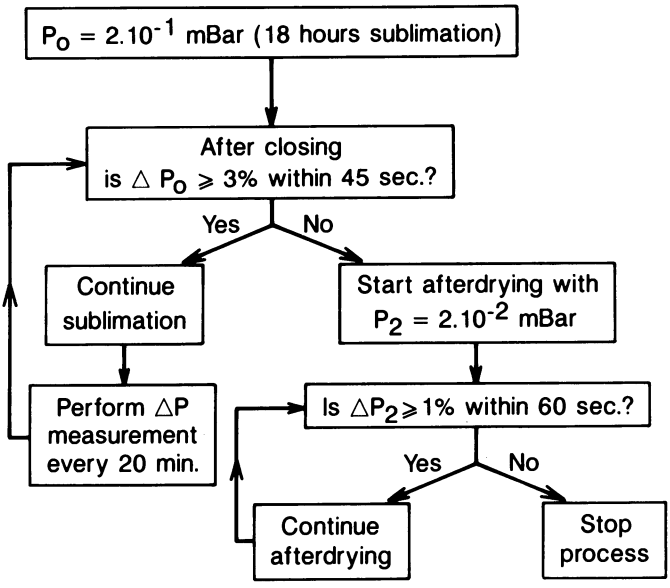

Figure 8. Pressure rise measurements with microprocessor/computer control during the sublimation period and the after-drying period. Intervals for subsequent pressure rise measurements as well as decision criteria have been indicated.

Control of the temperature of the sublimation interface is an important part of freeze-drying instrumentation (Fig. 7) equipped with computer control. Indirectly, this temperature can be determined by means of pressure rise measurements [16,17]. For automatic measurement, the drying chamber with the product is closed off from the vacuum pump during sublimation at certain intervals; then, the pressure P_0 rises (P_0) up to an equilibrium value P_1. Subsequently, the temperature of the sublimation interface can be deduced from P_1 by means of the P-T-diagram. Figure 8 gives an example of this control procedure. Intervals for subsequent pressure rise measurements as well as decision criteria have been indicated in this figure. Optimalisation of the parameters given in Figure 8 can be performed empirically. It is the expectation, that the importance of computer control will mainly reside in the higher reproducibility, with which successive batches of product can be prepared. In addition, control of the different quality influencing factors – like sublimation temperature, residual moisture content and partial water vapour pressure above the product – is thus better realized.

Another control of the freeze-drying process is the continuous measurement of the electrical conductivity of the frozen mass: when the electrical conductivity exceeds a predetermined threshold value, e.g. due to undesired temperature increase (melting effects), the product temperature is maintained below the critical temperature ($<T_e$). As a result, the sublimation process remains under control and the original physical structure is maintained (see Phase transitions).

In Figures 9a and 9b photographs of a freeze-drying chamber and of a vacuum lock installed upon a freeze-dryer are shown. With the help of vacuum lock samples can be withdrawn under vacuum for analysis without disturbing the process; with the analysis results a number of (experimental) possibilities exist:

- freeze-drying to a pre-determined, optimal moisture content
- shortening of existing cycles
- localize the cause(s) of bad reconstitution within sublimation or secondary drying phase
- localize the loss of biological activity within sublimation or secondary drying phase

It is evident, that the potential of a vacuum lock is enormous.

In Figures 10 and 11 examples of the use of a vacuum lock are shown: samples (vials) have been withdrawn from the shelf under vacuum during sublimation and the vial contents have been subsequently analyzed on moisture with a Mitsubishi moisture analyser. The decreasing residual moisture content as a function of time under vacuum (hours) can thus be monitored closely. This has advantages, when a pre-determined, optimal moisture content exists or when freeze-drying should proceed until the residual moisture content falls within the specification limits given to that particular product.

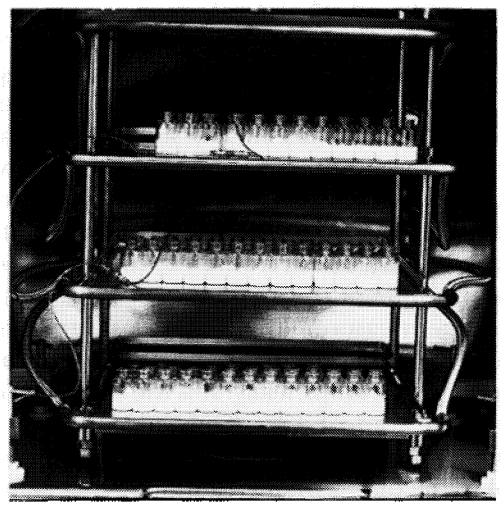

Figure 9a. Freeze drying of vials onto plates in a drying chamber with a diameter of 80 cm. This chamber can be closed with a perspex door. The wires are thermocouples, with which the temperature *within* the product is measured. Cooling liquid circulates through the metal pipes to the plates (inside hollow). In principle, the vials can be closed automatically under nitrogen (inert) atmosphere. In the upper part of the photograph, a part of the hydraulic cylinder is visible, which can move the plates towards each other for this purpose. The closing valve for pressure rise measurements is not visible.

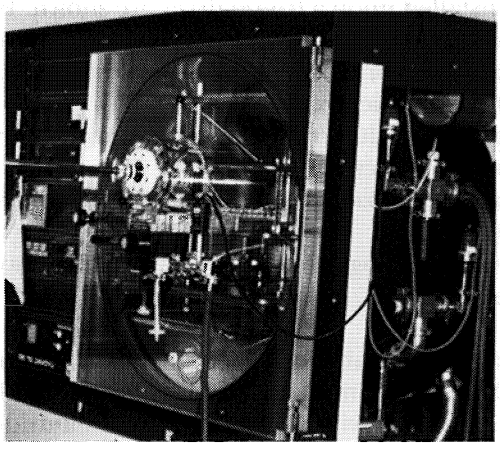

Figure 9b. Vacuum lock installed upon the perspex door of a GT20 freeze-dryer (Leybold Heraeus GmbH). The manipulator arm, with which samples can be with drawn, is visible perpendicular to the door. After withdrawal from the shelf, a vial is placed into the lock area under vacuum, closed with a rubber stopper, subsequently processed through a valve system at the bottom side of the lock and taken out for analysis.

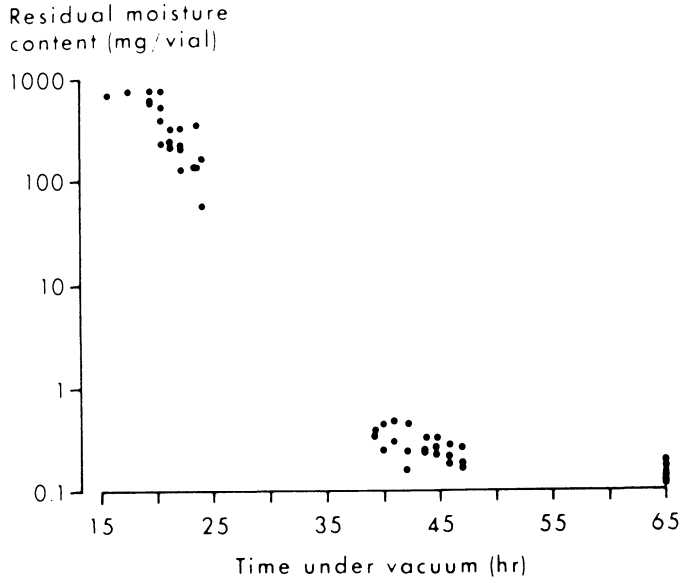

Figure 10. Residual moisture content measured during sublimation as a function of the time under vacuum. For moistere analysis, vials (73,5 mg lactose/vial) have been withdrawn under vacuum by means of the vacuum locks.

A minimum residual moisture content is not always the best solution. For a good stability of proteins after freeze-drying a certain residual moisture content is often required. This can be explained as follows: the strongly protein-bound water molecules lower the free energy content of the protein, whereas removal of these water molecules just increases the free energy content and destabilizes the protein.

On the other hand, a high residual moisture content also leads to destabilization (see Introduction).

A moisture *optimum* indeed exists in freeze-dried cells and protein preparations; the general shape of the curve is given in Figure 12 [18,19]. Ishibashi et al studied the significance of water activity of freeze-dried food ingredients and showed that the water activity is a decisive factor for the survival of freeze-dried bacterial cells. In addition, they studied the effect of the temperature, at which reconstitution/rehydration occurs, on the recovery of these cells [18]. The gross morphology, which the compound obtains after removal of ice/moisture, is sometimes characteristic: after sublimation of the ice crystals imprints imposed on the structure can be left.

Figure 13 shows a SEM photograph of a product, taken after freeze-drying: imprints with the same shape of the ice crystals and left as capillaries are clearly visible.

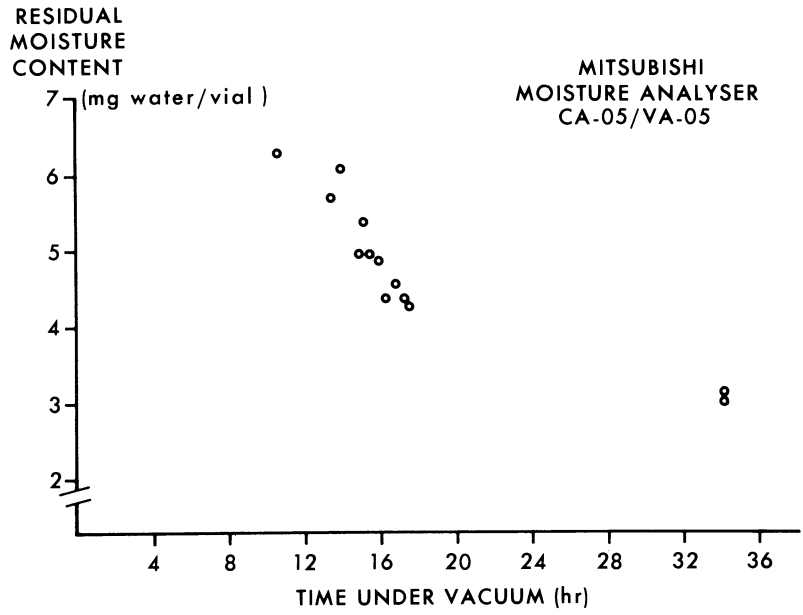

Figure 11. Residual moisture content measured during sublimation as a function of time under vacuum. For moisture analysis, vials (49 mg mannitol/vial) have been withdrawn under vacuum by means of the vacuum lock.

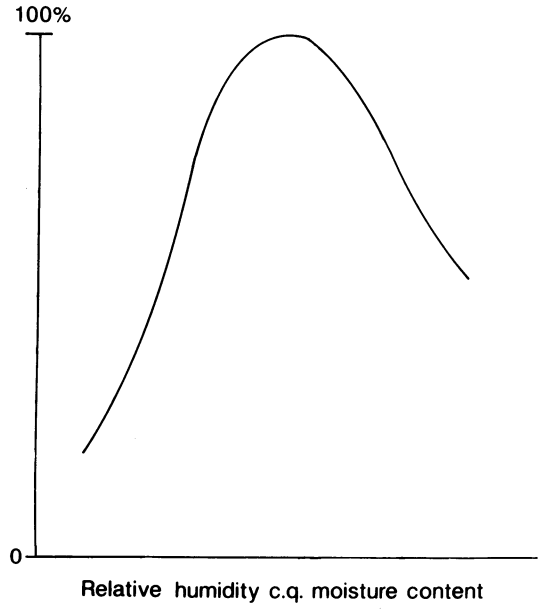

Figure 12. General shape of the curve, relating residual moisture to activity after freeze-drying.

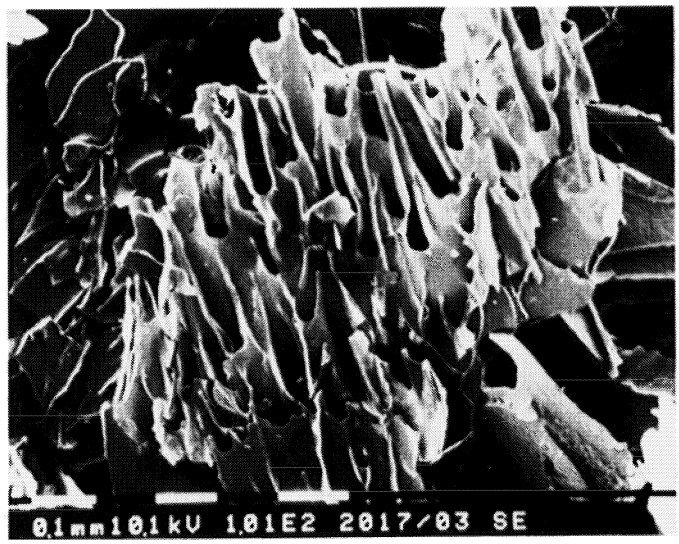

Figure 13. SEM photograph of corticosteroid after freeze-drying. The freezing temperature was –60°C.

Cryopreservation of nucleic acids by freeze-drying

Secondary and tertiary structure of nucleic acids

Transfer RNA is the smallest cellular RNA in both bacteria and eucaryotic cells. The molecule has a manifold of biological functions, among others its role in the decoding of the genetic message is perhaps the most remarkable. Decoding of the genetic message by transfer RNA (tRNA) plays an important role during the production of new products, including modern medicines in recombinant DNA technology. Consequently, it will be evident that the structure of these nucleic acids has gained considerable attention in recent biophysical studies.

Transfer RNAs have a molecular weight varying between 25,000 and 30,000 daltons. The molecules consist of a single polynucleotide chain of about 75 nucleotides. Although the primary sequence of these tRNAs may vary strongly, it turns out that they can be folded into a so-called cloverleaf structure thereby forming four double helical stacks containing the normal Watson-Crick AU and GC base pairs and occasionally a GU base pair [20].

X-ray diffraction studies and ^{1}H NMR studies confirmed the presence of the cloverleaf structure in the crystal as well as in solution [21]. The four double helical regions formed in the cloverleaf structure, i.e. the

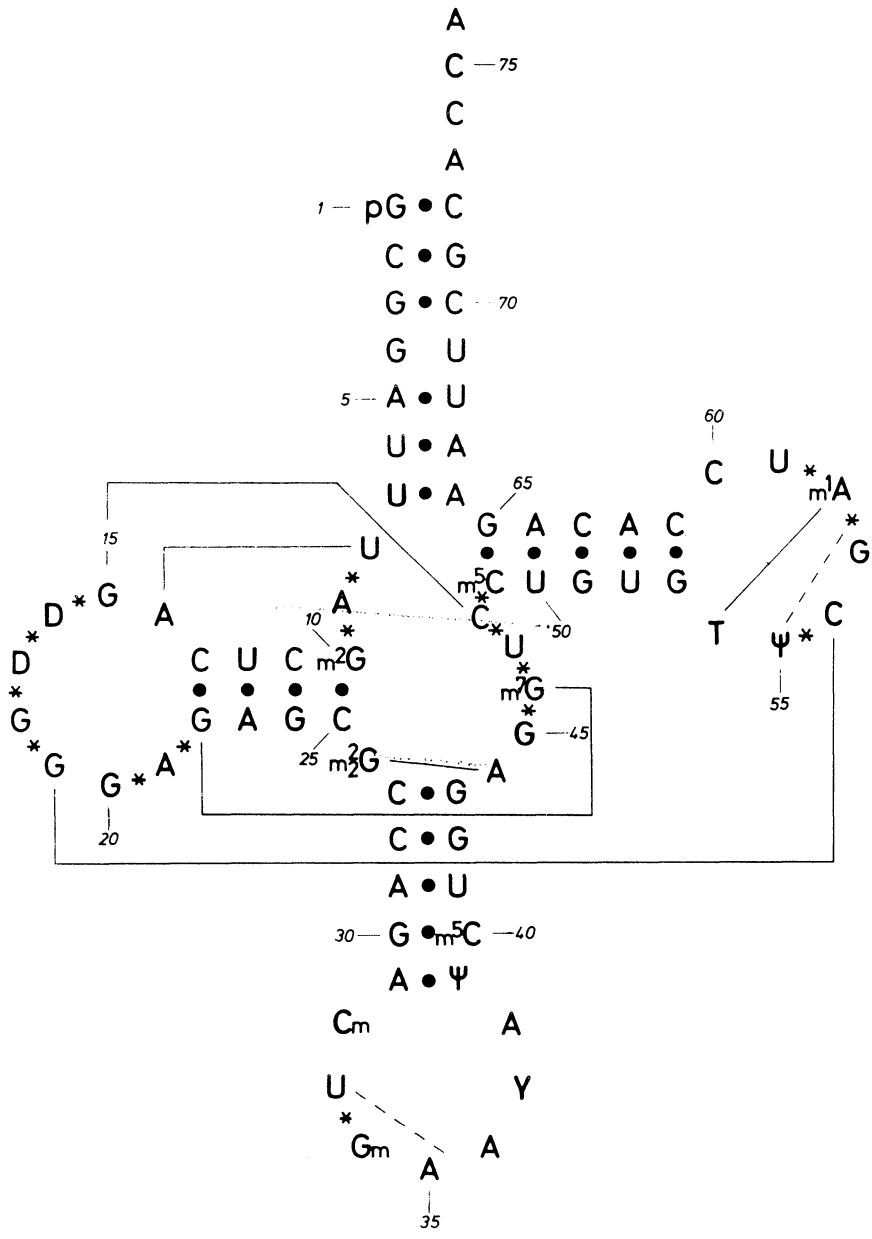

Figure 14. Cloverleaf structure of yeast $tRNA^{Phe}$ as determined by Raj Bhandary & Chang [43]. Asterisk indicate diesters with a deviating geometry [44]. The dashed lines represent two base – phosphate interactions; the solid lines represent tertiary hydrogen – bond interactions.

DHU (D), TψC, anticodon, and acceptor stem are indicated in Figure 14. The anticodon and TψC stems are connected by a variable loop, which may vary in length. The DHU and TψC loop, which are far apart in the secondary structure, are connected by hydrogen bonded pairs in the 3-dimensional structure.

In addition, in the 3-dimensional structure base pairs are formed between the DHU loop and the variable loop and between the DHU stem and the variable loop. The formation of these interactions is accompanied by a specific folding of the sugar phosphate backbone, which results in a L-shaped form of the molecule [21]. In the double helical parts of the molecule the backbone has the standard conformation found in normal RNA double helices. At the bends in the loops and in the nucleotide regions involved in tertiary structure, strong deviations from this conformation are found.

In the isolation and preservation of nucleic acids the ultimate aim is to process these biomolecules through the complete number of chromatographic and freeze-drying runs (Fig. 15) without damaging their secondary and tertiary structure. The integrity of secondary and tertiary structure can well be established with ^{1}H NMR and ^{31}P NMR respectively [9].

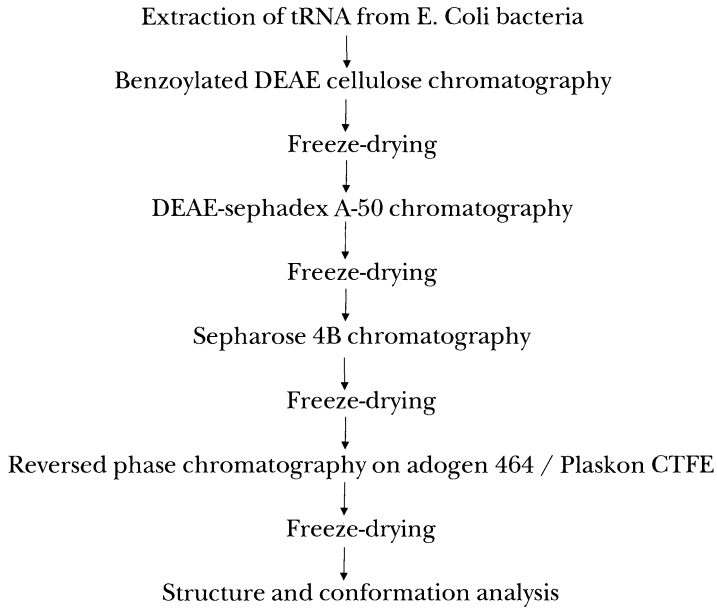

Figure 15. A schematic representation of the purification process of tRNA, in which preparative – large scale – liquid chromatography is interchanged with subsequent freeze-drying steps.

Below, the preservation of tertiary structure of tRNA after multiple chromatography and multiple freeze-drying is described.

^{31}P NMR and cryopreservation of nucleic acids

In nucleic acids the sugar phosphate backbone plays an important role in maintaining the correct folding of the molecular structure. It has been shown that the ^{31}P chemical shift is sensitive to the conformation and to the ionization state of the phosphate group, to complex formation with metal ions and to the character and structure of chemical substituents bound to the ester oxygens. For instance, an empirical correlationship between the O-P-O bond angle θ and the ^{31}P shift has been found.

From X-ray diffraction data on nucleic acids it follows that changes in the bond angle θ are accompanied by concomitant changes in the torsional angles W^1 and W. Therefore, a relationship between the ^{31}P shift and these torsional angles is also very likely. The X-ray diffraction data on the folding of the phosphate backbone in tRNA, together with the relationship between ^{31}P shifts and diester phosphate conformations discussed above, suggest that ^{31}P NMR spectra of tRNA may contain well resolved resonances, arising from special diester phosphate groups. Indeed, it was demonstrated that ^{31}P spectra of tRNA recorded at 40,5 and 100 MHz contain well resolved resonances. Consequently, these

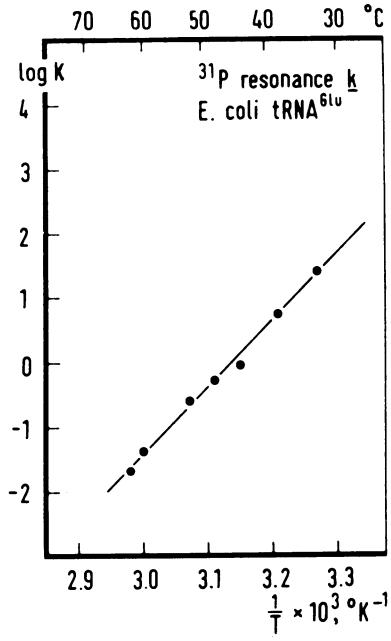

Figure 16. A v/h Hoff plot, recorded after four times freeze-drying; log K is plotted versus 1/T. The data refer to one specific ^{31}P resonance (k) in the spectrum of glutamic acid specific tRNA from E. coli.

resolved ^{31}P resonances can be used to monitor the preservation of the tertiary structure after freeze-drying (freezing rate: 40°C/min; cycle: 24 hours).

A v/h Hoff plot, based upon ^{31}P NMR data, is shown in Figure 16: Log K (equilibrium constant) is plotted versus 1/T (reciprocal absolute temperature). From the slope of the line in Figure 16 an enthalpy value of 206 kJ/mole is calculated.

Arrhenius plots, based upon ^{31}P NMR data, are given in Figure 17: τ (reciprocal reaction rate constant) is plotted versus 1/T. From the slope of the lines in Figure 17 Arrhenius activation energies of 176-353 kJ/mole are calculated. In view of the known enthalpy and activation energy values associated with the native tertiary structure of tRNAs, the conclusion is justified that multiple freeze-drying of tRNA leaves the tertiary structure mainly intact under properly chosen freeze-drying conditions.A ^{31}P NMR spectrum, obtained from yeast $tRNA^{Phe}$ with preserved tertiary structure (folded), is given in Figure 18. The ^{31}P NMR signals, arising from the tertiary structure, have been indicated. In addition, in this figure a ^{31}P NMR spectrum without preserved tertiary structure (unfolded) has been given: the ^{31}P NMR signals, arising from the tertiary structure, have disappeared now. This figure demonstrates that preservation or loss of tertiary structure can indeed by closely monitored with ^{31}P NMR.

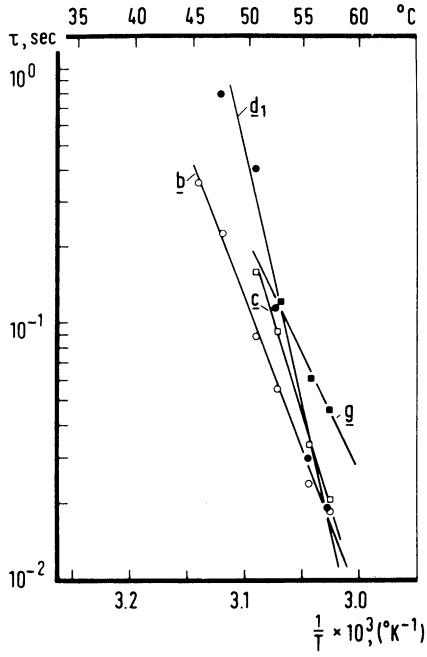

Figure 17. Arrhenius plots, recorded after fourtimes freezedrying; is plotted versus 1/T. The data refer to fourspecific ^{31}P resonances in the spectrum of phenylalanine specific tRNAfrom yeast.

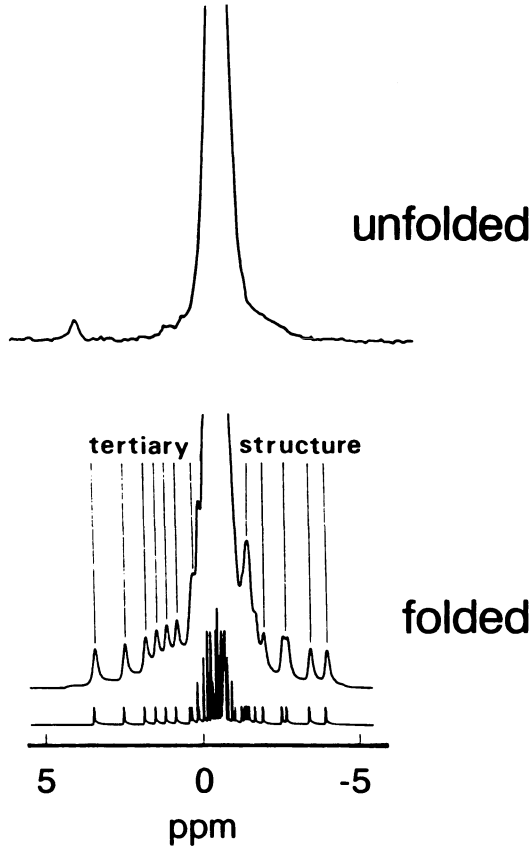

Figure 18. ^{31}P NMR spectra of yeast tRNA^{Phe} with (folded) and without retention of tertiary structure (unfolded). The ^{31}P NMR signals arising from tertiary structure have been indicated.

Cryopreservation of synthetic cells by freeze-drying

Moisture: transport through the cell membrane

Evidently, the freeze-drying process of isolated and purified biopolymers does not mimic the freeze-drying process of these biopolymers while being present in their natural cell environment. In the latter case, it can be expected that structure preservation is also triggered by transport phenomena through the cell membrane occurring during freezing as well as during freeze-drying/rehydration. Next to the integrity of the biopolymers, in the presrvation of structural and functional integrity of cells residual water left after freeze-drying is crucial. It is well-known, that transport of solute(s) and/or solvent during freezing can be described

by the phenomenological equations of Onsager [22]. It remains to be established, whether extension to freeze-drying introduces an additional term (the hydrostatic pressure gradient) into these equations, as compared to the case with only freezing.

The tendency to maximal disorder or maximal entropy can be considered as the driving force behind the leakage of solutes/molecules from cells, subjected to freezing/freeze-drying. The magnitude of this driving force can be expressed in the dissipation function Φ described by Rayleigh [22].:

$$\Phi = \sum_{i=1}^{N} \text{flow x driving force}$$

In this dissipation function several distinct terms can be present. For instance, when considering a (temporary) temperature gradient and an osmotic pressure gradient over the cell membrane during freezing as well as a (temporary) hydrostatic pressure gradient during sublimation under vacuum, the dissipation function Φ would be given as:

$$\Phi = J_u \bullet \Delta T + J_{vol} \bullet \Delta P + J_{diff} \bullet \Delta\pi$$

in which:
J_u = heat flow; ΔT = temperature gradient; J_{vol} = volume flow; ΔP = hydrostatic pressure gradient; J_{diff} = diffusion flow; $\Delta\pi$ = osmotic gradient.

From this equation, it is seen that the dissipation function is built up from three terms, i.e. the three gradients (=driving forces) multiplied by their own (=conjugate) flow.

According to Onsager, each flow is in principle determined by *all* gradients (driving forces), operating upon the cell. Thus, for processes which occur sufficiently slowly, the flow is linearly related to all gradients. Consequently, a coupling may exist between a gradient of one type and a flow of another type. On the basis of the equation given above, the following expressions can be deduced, describing the leakage/flow of cellular water of dissolved compounds.

For convenience, it has been assumed in this example that $\Delta T=0$:

$$J_{vol} = L_{11} \bullet \Delta P + L_{12} \bullet \Delta\pi$$
$$J_{diff} = L_{21} \bullet \Delta P + L_{22} \bullet \Delta\pi$$

in which:
L_{11} = filtration coefficient; L_{12} = osmotic flow coefficient; L_{22} = diffusion coefficient; L_{21} = ultrafiltration coefficient.

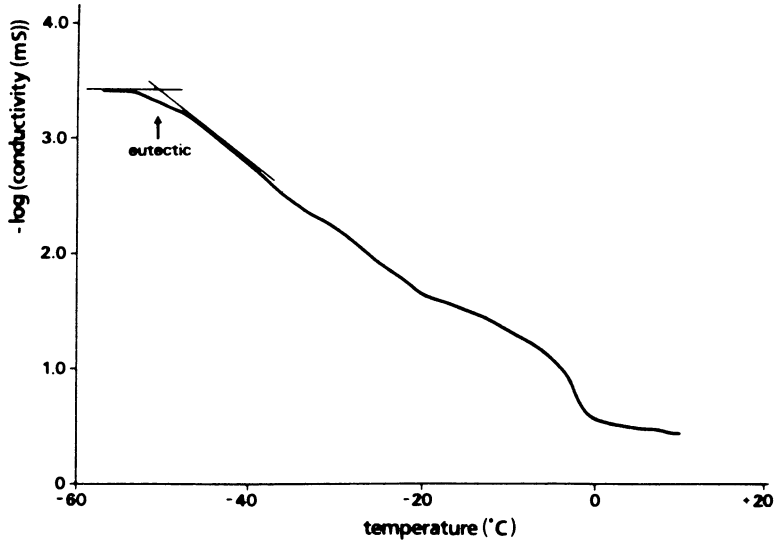

Figure 19. The conductivity (mS) of the liposome dispersion as a function of temperature is an aqueous solution containing 10% (v/v) glycerol, 10% (m/v) mannitol, 100 mmol/l sodium chloride and 10 mmol/l Tris, pH 7.4 [6].

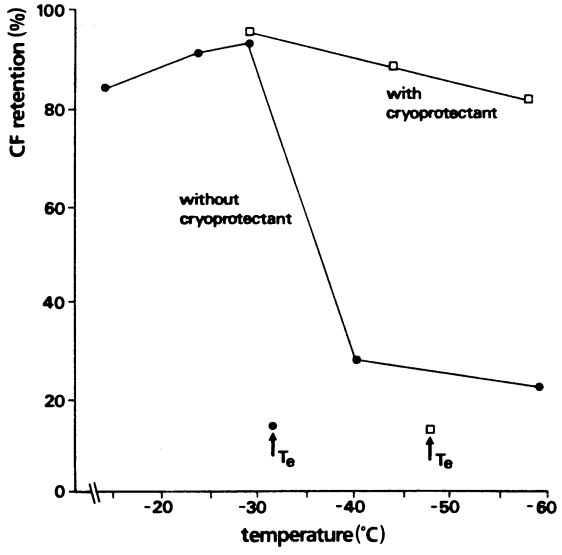

Figure 20. The influence of freezing temperature on CF retention (%) after a freezing/thawing cycle with (❑) and without (•) a cryoprotectantmixture (10% glycerol, 10% mannitol). The positions of Te (eutectic temperature) are indicated. Freezing time, 20 min; freezing rate7°C min^{-1} [6].

The coefficients L_{11} and L_{22} are the so-called "straight" coefficients; these give the relations between the flow and its own conjugate gradient (force).

The coefficients L_{12} and L_{21} are the so-called "cross" coefficients; these give the relation between the flow and a non-conjugate gradient (force). If these coefficients are equal to zero, then there is no relation between a gradient of one type and a flow of another type.

If $L_{21} \neq 0$, the hydrostatic pressure gradient yields *not* only a volume flow, but *also* yields a diffusion flow across the cell membrane. The magnitude of the coefficients (ml/dyne. sec. cm^2) depends upon the type of cell membrane.

To summarize, it follows from this approach in the irreversible thermodynamics that many changes accomplished with a cell can result in diffusion/leakage of a compound from the cell, because the "cross" coefficients are not equal to zero.

Liposomes as synthetic cells

Liposomes can be considered as synthetic cells, consisting of a phospholipid bilayer, and mimic peripheral blood cells in different ways. Liposomes and phospholipid systems have been extensively studied in cryopreservation studies, i.e. freezing / thawing and freeze-drying / rehydration [23-41]. Advantages of liposomes as a model system are: easy availability, uniformity of cell size, own choice of lipid composition and contents simplicity. The integrity of liposomes after freeze-drying depends upon freezing temperature, freezing rate, freezing time, the presence of cryoprotectants and the eutectic temperature, among other factors. During development of a freeze-drying cycle for liposomes, attention should be given to minimize size distribution changes, leakage and deterioration of cellular compounds as well as lipid degradation.

Liposome integrity can be preserved in the presence of properly selected cryoprotectants with respect to stability against aggregation, fusion and membrane damage due to intra liposomal ice crystal formation, osmotic

Table 2. Eutectic temperatures (T_e) calculated from conductivity measurements. Liposomes: PC-H/DCP (10/1), MLV; pH 7.4 [6].

	T_e (°C)
Liposome dispersion (100 mM NaCl, 10 mM Tris)	–33
Internal phase liposome (50 mM CF, 10 mM Tris)	–37
External phase (100 mM NaCl, 10 mM Tris)	–29
Liposome dispersion (100 mM NaCl, 10 mM Tris 10% glycerol – 10% mannitol)	–48
Liposome dispersion (100 mM NaCl, 10 mM Tris 10% glycerol – 10% lactose)	–48

dehydration and/or high ionic strength. Leakage of lipophilic, bilayer associated molecules is low on rehydration of the freeze-dried product, whereas leakage can be substantial for hydrophilic, non-bilayer interacting molecules.

Recently, it was hypothesized that the eutectic temperature plays an important role in the cryopreservation of liposomes [6]. In Figure 19 an example of a conductivity curve recorded on a liposome dispersion is given. In this case, the eutectic temperature was determined by measuring the intersection of the tangents to the curve in the low conductivity region as indicated in Figure 19. The eutectic temperature thus calculated is given in Table 2, together with those of a number of aqueous solutions and dispersions. In Figure 20 the leakage of carboxyfluorescein (CF), a hydrophilic non-bilayer interacting compound, from liposomes is given as a function of freezing temperature after a freezing/thawing cycle with and without 10% glycerol/10% mannitol as the cryoprotectant mixture. Leakage has been expressed as % CF retention within the cells. Below –25°C, this cryoprotectant mixture reduced leakage as compared to the case without cryoprotectants. Note also the influence of T_e indicated in this figure [6].

In their study on liposome-carbohydrate interactions Crowe et al. compared various carbohydrates (maltose, trehalose, sucrose and lactose a.o.) in their ability to prevent leakage of isocitrate as the intraliosomal compound during freeze-drying [42]. They found, that the mass ratio of carbohydrate:lipid rather than the molarity of the solution is an important parameter for prevention of lipid mixing and leakage. Especially, trehalose, a non-reducing disaccharide, is promising in this respect: retention of isocitrate as the marker compound was near 100% in freeze-dried liposome/trehalose preparations.

Cryopreservation of proteins by freeze-drying

Proteins compose a class of biopolymers, to which the interrelationships between freeze-drying parameters/variables as given in Figure 1 also certainly apply. Many parameters/variables exert effects upon each other. Often, during freeze-drying of protein solutions no nucleation of solute occurs: this results in freeze-dried proteins with an amorphous structure, associated with potential problems with hygroscopicity, with activity loss, with reconstitution/solubility/opalescence and with collapse.

With collapse is meant the physical collapse of the freeze-dried cake. In this respect, a so-called critical collapse temperature may be defined, above which morphological changes easily occur. Exceeding this temperature by only 2-3°C can be enough to cause a strong reduction of the rate of rehydration after reconstitution (poor solubility). It is important to have knowledge of the collapse temperature in an early stage of product development. Collapse does not have to occur within all vials of a batch. Thus, collapse may be one explanation for the fact, that in a large

batch of vials e.g. 95% of the vials is successfully freeze-dried and 5% is not. A second explanation for the same situation (5% of the vials not successfully freeze-dried) can be the absence of nucleation of one or more of the solutes.

Loss of biological activity of a protein upon freeze-drying can result from several causes:
- evaporation of the active substance due to the absence of a proper binder
- overdrying by removing thermodynamically important water molecules from the protein (during desorption drying)
- partial melting during freeze-drying
- morphological changes of the cake, resulting in denaturation

Sometimes surface films, which in principle may vary in thickness, are seen after freeze-drying of proteins. Prefreezing by extraction of heat only via the bottom shelf is an important factor, which causes the generation of surface films. This problem can be anticipated by prefreezing, while air is gently blown through the chamber; this results in heat removal, which is more uniform in all directions.

As mentioned previously in this paper, often an optimum moisture content exists in freeze-dried protein preparations. It is finally noted here, that moisture contents should be envisaged in several ways:
- optimum moisture content/mg of protein
- uniformity of moisture throughout all vials of a batch
- uniformity of moisture within a single vial (the upper part of a vial can be in secondary drying, whereas the bottom part is still in sublimation)

Conclusions

From the foregoing discussion it is concluded, that – in order to guarantee reproducible and reliable cryopreservation – it is indispensable to:
- recognize the physico-chemical and technical parameters, which affect the quality/activity of freeze-dried cells and constituents
- have knowledge of the quantitative interrelationships between these parameters

Acknowledgement

I like to acknowledge Mrs. H. Nelissen-Verhoeven, who has typed the successive drafts and the final version of this manuscript with patience and accuracy.

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FREEZING VERSUS VITRIFICATION; BASIC PRINCIPLES

D.E. Pegg, M.P. Diaper

The discovery by C. Polge, U.A. Smith and A.S. Parkes in 1948, that glycerol would enable fowl spermatozoa to survive freezing to –70°C [1], initiated a phase of dramatic development in the application of what subsequently came to be called "cryobiology". It is interesting to note, in the context of this paper, that the title of the paper describing that fundamental observation was "Revival of spermatozoa after vitrification and dehydration at low temperatures"; as we shall see, these experiments did not produce vitrification in the sense that is now meant – in fact, that method would now be termed the "classical freezing" approach. In this paper we shall consider the mechanisms by which the classical freezing method and vitrification seek to preserve the viability of cells, tissues and organs.

The success of classical freezing methods depends upon the addition of a cryoprotective compound such as glycerol, and the empirical optimization of a number of variables that have been shown, experimentally, to affect survival: these include the nature and concentration of the cryoprotectant and the temperature at which it is added, the rates of cooling and warming, the storage temperature and the temperature and rate at which the cyroprotectant is removed. Although this approach is entirely empirical it has been strikingly successful and effective methods were soon developed for a wide range of cells, including the spermatozoa of several species, erythrocytes, lymphocyte and haemopoietic cells, various endocrine cells and many strains of tissue culture cell: these early successes are well documented in A.U. Smith's 1961 monograph "Biological effects of freezing and supercooling" [2]. These practical successes stimulated a considerable volume of more fundamental work that has uncovered a number of the mechanisms that are involve – the fundamental importance of the total quantity of ice that is formed, the location of the ice crystals in relation to the cells, the toxicity of cryoprotectants and the temperature dependence of that toxicity, and the magnitude of osmotically-induced changes in volume. These factors will now be considered in more detail.

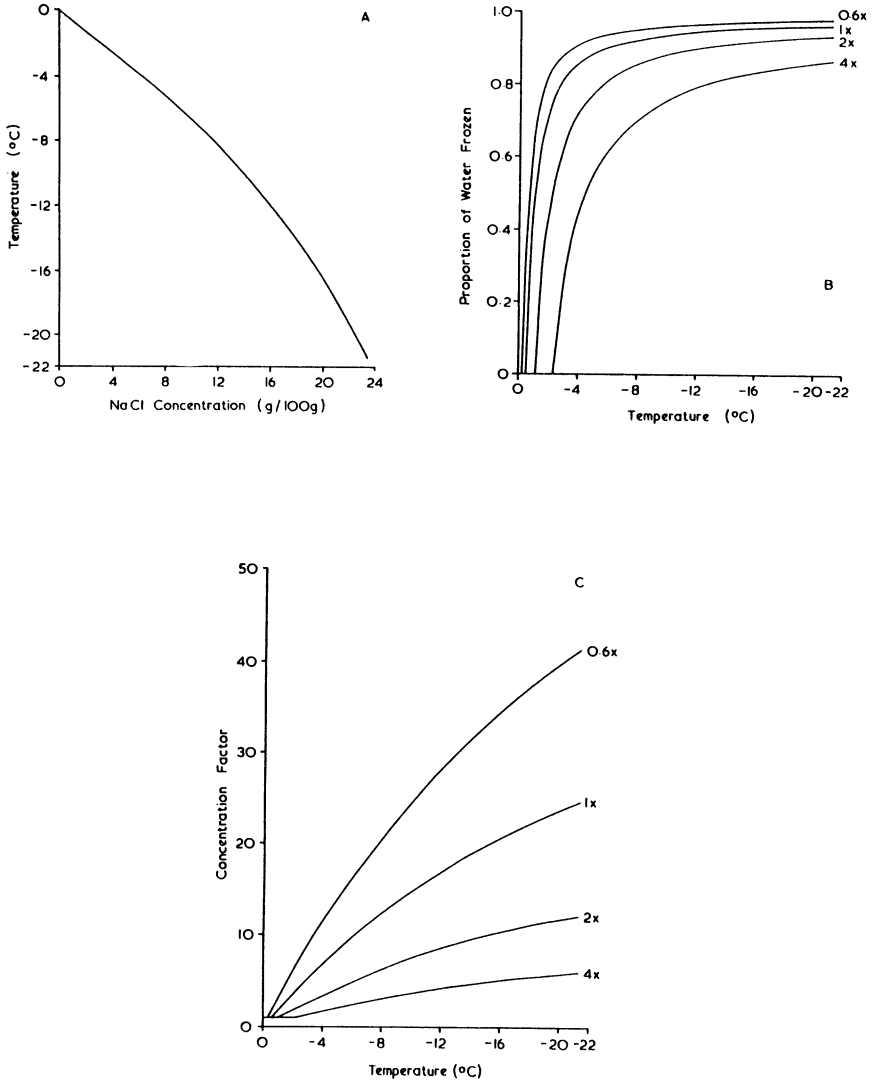

Figure 1. Panel (a) shows the equilibrium freezing point (liquidus) curve for the binary system NaCl/water.
Panel (b) plots the proportion of water that is converted to ice when four solutions of NaCl in water are progressively frozen: 0.6X signifies 0.6 times the isotonic concentration; 1× signifies the isotonic concentration which is taken to be 0.95/100g; 2× and 4× indicate solutions that are hypertonic by those factors.
Panel (c) plots the factor by which the concentration of NaCl increases as a consequence of freezing the same solutions that are shown in panel (b).

The role of ice

When a dilute aqueous solution is frozen, the ice that forms is essentially pure crystalline water; it has negligible ability to dissolve solutes. Solutes are therefore rejected and concentrate in the dwindling volume of unfrozen liquid, depressing the chemical potential of water and achieving equilibrium with ice at each temperature. Thus the familiar freezing-point depression curve of an aqueous solution (that for NaCl/water is shown in Figure 1a) describes the dependence of solution composition upon temperature. Note that, in the presence of ice and at equilibrium, composition is determined solely by temperature and is therefore independent of *initial* composition; however, initial composition does control the amount of ice that forms and the factor by which the concentration increases at a given temperature; this is illustrated for NaCl/water in Figures 1b and 1c. It will immediately be apparent that the increase in concentration of NaCl that occurs during the freezing of isotonic saline is enormous, actually 25-fold at –21°C. The situation is more complex in living biological systems but since the fundamental physical chemistry has to be the same, we can, as a first approximation, treat cells as microscopic semipermeable bags of isotonic saline immersed in isotonic saline. The question then arises: Is it the ice, the elevated salt concentration or both that damage cells during progressive freezing? A rather definite answer was given by Lovelock in a series of classical papers published in the early 1950s [3–6]. Lovelock showed that the extent of haemolysis observed in human erythrocytes that were frozen to various temperatures in solutions of sodium chloride could be accounted for quantitatively by the effect of the salt concentration produced by freezing to each temperature. A duplication of that experiment in our laboratory is illustrated in Figure 2, and does indeed show an impressive correspondence between the effect of the two treatments. To be rigorous however, it should be noted that an experiment of this sort cannot prove causation, although the correlation may, as in this case, convince many workers. Lovelock went on to explain the cryoprotective action of glycerol, and other highly-soluble, non-toxic, penetrating solutes by their ability to moderate the increase in salt concentration that occurs during freezing; this is demonstrated for the system NaCl/glycerol/water in Figure 3. This explanation of cryoprotection tended to reinforce acceptance of the "salt damage" theory of freezing injury, but as Mazur has pointed out [7], there is a fixed relationship between salt concentration and the amount of ice formed at any given subzero temperature when solutions of a given cryoprotectant in isotonic saline are frozen: since cryoprotectants actually moderate the rise in salt concentration by reducing the amount of ice, the existence of the phenomenon of cryoprotection does not support either the salt- or the ice-damage theory! We have argued that it would indeed be surprising if the correspondence between the effect of salt concentration and that of freezing were

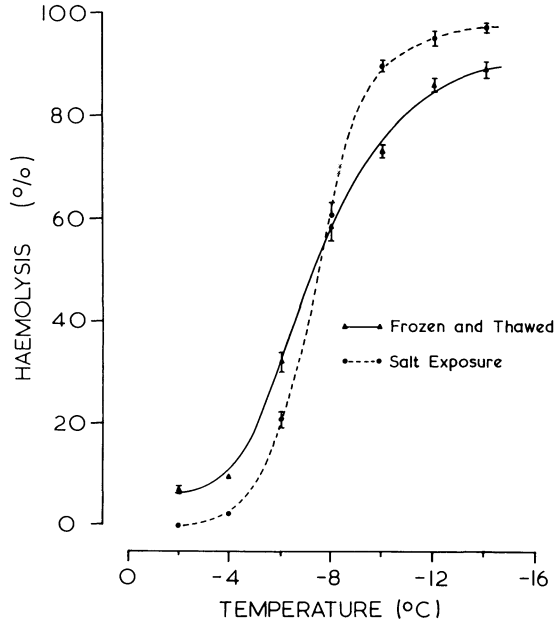

Figure 2. Experimental results when human erythrocytes were frozen to the indicated temperatures and then thawed (▲) compared with exposure to equivalent salt concentrations and return to isotonic conditions (●).
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merely coincidental, and when we found that the addition of glycerol had the same influence on the effect of exposure to high concentrations of salt as it did on the effect of salt-concentration by freezing [8], we considered it highly probable that the salt-damage theory was valid. Mazur however has provided extensive experimental evidence that ice may indeed have a direct damaging action [7,9,10,11]. This was done by freezing erythrocytes in solutions of glycerol and sodium chloride in which *the initial concentration of salt varied between 0.6X and 4.0X isotonic*, which made it possible to separate, to a considerable extent, the increase in salt concentration from the quantity of ice that formed. Mazur found a stronger correlation between severe damage and the amount of ice rather than the salt concentration.

One problem with this sort of experiment is that the means used to separate the variables (different initial tonicities) also affects the cells: cells destined to be subjected to low "unfrozen fractions" entered the experiment in a swollen state, and we have suggested that such cells may behave differently from normal cells [12]. In fact, we were able, with erythrocytes suspended in solutions of NaCl/glycerol/water, to demon-

strate remarkably similar responses to freezing and thawing as to exposure to equivalent concentrations of solute and redilution [8]. When similar experiments were carried out in the *absence* of glycerol, the correspondence between the effects of freezing and solution-exposure was good for cells in isotonic and 2× isotonic saline, but freezing was substantially more damaging than solution-exposure when the cells were suspended in 0.6× or 4× saline [13]. The mechanism of this effect is unclear, but it is consistent with the proposition that cells suspended in a range of tonicities of saline do not behave as a uniform population. At this stage we remain unconvinced that ice has any direct role in freezing injury to erythrocytes, but we do not exclude that possibility.

In the foregoing discussion we have modelled cells as microscopic semipermeable bags of saline, that is, the system is compartmentalized: it is important therefore to consider whether ice forms inside or outside the cells or both. Freezing is a nucleation-induced event, and the probability of nucleation increases directly with the degree of supercooling and with the volume. Thus, as our compartmentalized model cools below the equilibrium freezing point of the saline, it is inevitable that a nucleation event will occur in the single, large extracellular compart-

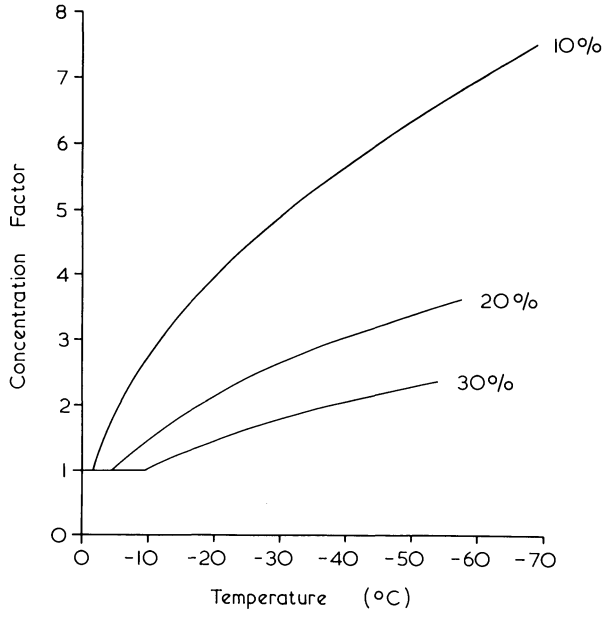

Figure 3. The effect of the concentration of glycerol (g/100g as indicated against each curve) on the proportional increase in solute concentration during progressive freezing. Each solution contained 0.95g/100g NaCl, so the ordinate indicates the factor by which the tonicity of salt in the solution increased at each temperature.

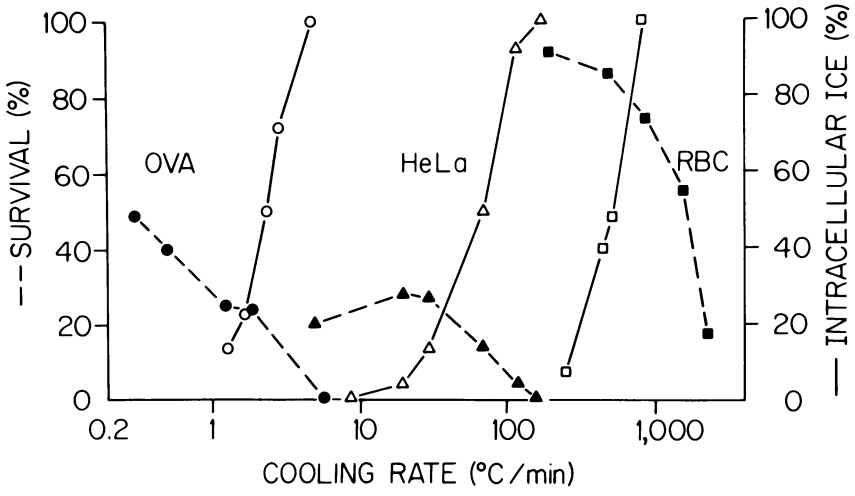

Figure 4. Survival (●) and the percentage of cells that froze internally (○) of mouse ova frozen in 1M dimethylsulphoxide, HeLa cells frozen in growth medium (▲, △), and red blood cells frozen in 1.5M glycerol (■, □).

ment before very many of the cells have nucleated: once that has happened, further cooling will result in growth of that extracellular ice, concentration of the extracellular solution and dehydration of the cells by osmosis through their semipermeable membranes. Thus, providing the cooling rate is sufficiently low and the water permeability of the cells sufficiently high the intracellular spaces will remain free of ice. This fundamental phenomenon was first studied by Mazur [14] who provided a quantitative analysis of the effect of cooling rate on water transport during progressive cooling, and correlated the predicted extent of intracellular supercooling with the known effect of cooling rate on cell survival: the greater the degree of supercooling the greater the probability of freezing. It was shown that cooling rates that produced significant supercooling with each cell also caused the survival to fall. The phenomenon is best illustrated by the data of Figure 4, compiled by Leibo [15] which shows, for three types of cell with differing water permeabilities, an inverse correlation between intracellular freezing and survival. Thus we conclude that extracellular ice is probably innocuous to cells in suspension, whereas intracellular ice is generally lethal.

This discussion has dealt with a grossly simplified model of cells in suspension and the simplest of all mammalian cells (the erythrocyte), together with a few examples of more typical mammalian cells in suspension. But many of us would like to be able to cryopreserve more complex systems – multicellular, organized tissues and organs. Several factors

conspire to make this a vastly more difficult task – the fact that tissues consist of many types of cell that may differ in their requirements for optimal preservation, the restriction of rates of cooling and heating that the given dimensions of the tissue or organ imposes, and above all, the fact that the function of an organ or tissue depends upon the interrelations between the cells – their connections, the preservation of an adequate 3-dimensional arrangement and the integrity of non-living intercellular structures such as basement membranes, glycosaminoglycans and collgen fibres. The point to be emphasized is that ice forming outside the cells may nevertheless be within the system we wish to preserve and may therefore produce lethal injury. In recent discussions of freezing injury in tissues and organs, we have concluded that damage due to extracellular ice is the single most serious obstacle to the extension of cryopreservation techniques to multicellular systems [16,17]. In the case of vascularized tissues, and particularly of organs, we have obtained both experimental and theoretical evidence for the crucial role of intravascular freezing: rabbit kidneys, frozen after equilibration with 2M glycerol, were found to have ruptured glomerular capillaries [18]; mathematical modelling of the process of freezing in a Krogh cylinder model of vascularized tissue showed that the capillaries would have to expand 8-fold to accommodate the required volume of ice – far beyond their elastic limit [19].

We conclude that ice is exceedingly damaging, by a number of mechanisms, to tissues, even in the presence of concentrations of cryoprotectant that would ordinarily (that is, with a typical cell suspension) be expected to provide a high degree of protection. The route to effective cryopreservation of such systems would therefore seem to be via ice-free cooling, or vitrification. The term "vitrification" must now be defined explicitly for aqueous systems: it is the conversion of the system from a fluid to a solid solely by an increase in viscosity, without a phase-change, without any crystallisation of water, and therefore in the complete absence of ice. It will be the principal purpose of the remainder of this paper to explore means of attaining this state in viable systems, but first we shall consider the two other factors mentioned in the introduction, chemical toxicity and osmotic damage: these are important in conventional freeze-preservation, but they acquire an added importance in vitrification because the concentrations of added solute are much greater.

Toxicity of cyroprotectants

Cryoprotectants are of necessity tolerate din high concentrations; glycerol is, in this sense, far less harmful than NaCl, but at a sufficiently high concentration any compound will be "toxic". This has two important consequences: the highest concentration that the tissue will tolerate *prior* to preservation is limited; and *during* freezing, the concentration increases as ice separates. In vitrification, as opposed to freezing, a much

higher initial concentration is needed but no further concentration occurs during cooling since freezing does not occur. In both techniques one seeks the highest tolerable concentration, in freezing to reduce the salt concentration, and in vitrification actually to achieve the vitreous state. In practice it is found that the maximum concentration that can be achieved without impairment of viability is dependent on the temperature and rate of addition and removal; temperature-dependence is due partly to osmotic effects (damage is increasing by reduction in temperature) and partly to chemical toxicity (damage is reduced by reduction in temperature). Osmotic effects will be discussed later. Cryoprotectants are frequently added at reduced temperature in order to take advantage of the positive temperature coefficient of chemical toxicity, usually at 0-4°C rather than at room temperature, but this factor has assumed a far greater importance in attempts at vitrification. Thus, for the vitrification of mouse embryos, Rall and Fahy used a 2-stage incubation with their cryoprotectant mixture, first at 20°C with one quarter of the final concentration and then at 4°C with the final concentration [20]. Much earlier than this, Elford and Walter [21] increased the concentration of dimethylsulphoxide in smooth muscle to a final level of 50% w/v by a series of step increases at 37°C (to 20%), –7°C (to 30%), –14°C (to 40%), –22°C (to 50%), –39°C (to 60%) and the muscles were then cooled to –79°C. Reversal of this process during warming permitted full recovery of contractile function, and no ice was formed at –79°C. Thus, in this sytem, a very high concentration of cryoprotectant was tolerated when the temperature, and rate of addition and removal were appropriately optimized. Empirical experiments in our laboratory have shown that rabbit kidneys will tolerate up to 4M glycerol [22], while rabbit corneas would recover after exposure to 4.25M dimethylsulphoxide [23]. Unfortunately, the concentrations of cryoprotectant needed to vitrify aqueous systems are generally in excess of 5.5M, which is usually beyond achievement. With some cryoprotectants, even during conventional freezing methods of cryopreservation, it seems that toxic levels are reached: this was first pointed out by Lovelock in his study of the cryopreservation of erythrocytes with methanol, when it was observed that irrespective of the initial methanol concentration, recovery fell to negligible levels when the temperature was reduced below –55°C. With some systems a similar, if less severe phenomenon occurs with propan-1,2-diol (propylene glycol); for example with human platelets [24] and rabbit kidneys [25], but other systems, that include rabbit cornea [26] and human embryos [27], seem to tolerate this cryoprotectant more readily. It seems very likely that all cryoprotectants, at the concentrations used in practice, produce some damage – certainly this is true of glycerol and human erythrocytes [8] – but where the additive is effective this toxic action is clearly less than the cryoprotective effect. The crucial points are that there always is a toxic limit to the concentration of cryoprotectant that can be used, and that

the apparent toxic limit is strongly influenced by the conditions under which the cryoprotectant is added and removed.

Osmotic effects of cryoprotectants

The most effective cryoprotectants penetrate cell membranes, but they do so more slowly than water, which means that some osmotic imbalance is inevitable during the addition or removal of these compounds. Gross osmotic shock results in cell damage, ultimately in lysis, and it is there-

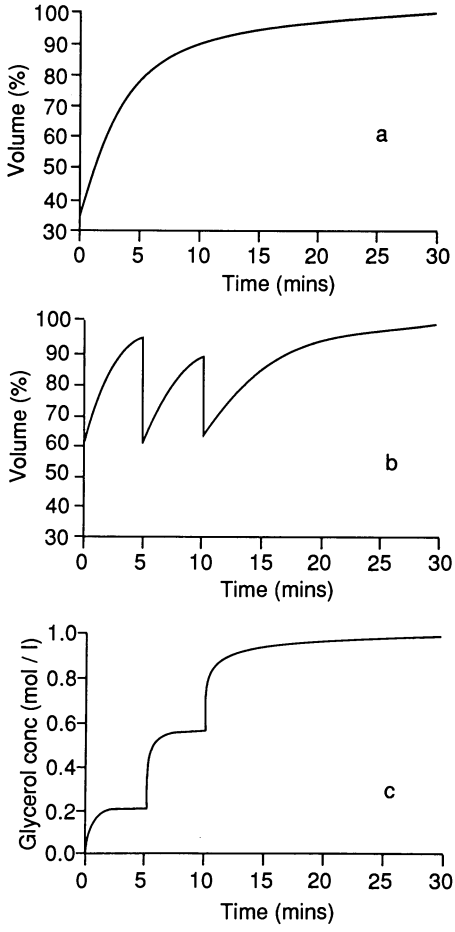

Figure 5. Calculated changes in cell volume and intracellular glycerol concentration during the addition of glycerol to platelets.
(a) Addition of 1M glycerol as a single step. (b) Addition of glycerol in three steps: 0.25M, 0.6M, and 1.00M. (c) Intracellular glycerol concentration in procedure (b). (Reproduced with the permission of Academic Press.)

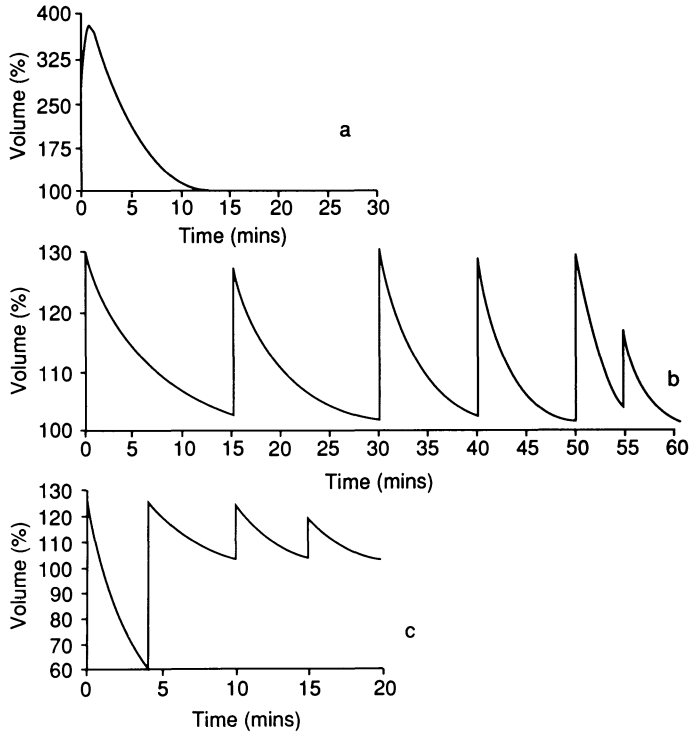

Figure 6. Calculated changes in platelet volume during removal of 1M glycerol. (a) Dilution to zero glycerol concentration in a single step. (b) Dilution to zero glycerol concentration in 6 steps using an isotonic diluent (0.7, 0.5, 0.31, 0.17, 0.05, and 0.00M). (c) Dilution to zero glycerol concentration using an initial dilution with a hypertonic solution followed by a dilution step with water to restore isotonicity and finally two isotonic dilution steps. Note that method (c) requires fewer steps and is completed more speedily. (Reproduced with the permission of Academic Press.)

fore both logical and of proven efficacy to control changes in cell volume so that acceptable limits are not transgressed. An important step in designing cryopreservation methods is therefore to measure the volume-response to changes in external osmolality of a nonpermeating solute and to correlate each volume with subsequent structure and function. The next step is to measure, or failing that the estimate, the water and solute permeability and the solute reflection coefficient for the chosen cryoprotectant. Two stratagems for controlling volume during the addition and removal of penetrating solutes are available: the first is to use low rates of change in concentration or, what amounts to the same thing, change the concentration in several small steps; the other approach, which is applicable only to the removal phase (which is the more

dangerous phase), is to incorporate solutes into the solution that do not penetrate the cells and therefore function as "osmotic buffers" by restricting the inflow of water as the external concentration of cryoprotectant is reduced. These highly effective procedures are illustrated for the addition and removal of glycerol with human platelets in Figures 5 and 6. Such processes are even more important when concentrations of penetrating agents sufficient to achieve vitrification are used.

Vitrification of cells, tissues and organs

Vitrification occurs when the viscosity of the solution reaches a sufficient value (arbitrarily set at $10^{14.6}$ poises) and crystallization of ice is inhibited. Since the concentration of solute in the reamining liquid phase increases during progressive freezing, a temperature will eventually be reached with many systems at which that residual liquid vitrifies *in the presence of ice.* It has already been pointed out that, under the cooling conditions that are usually used, ice does not form inside the cells, and particularly since intracellular protein promotes vitrification, it is actually the case that the *cells* in conventionally-frozen material are vitrified: Polge, Smith and Parkes were not, after all, in error in the title of

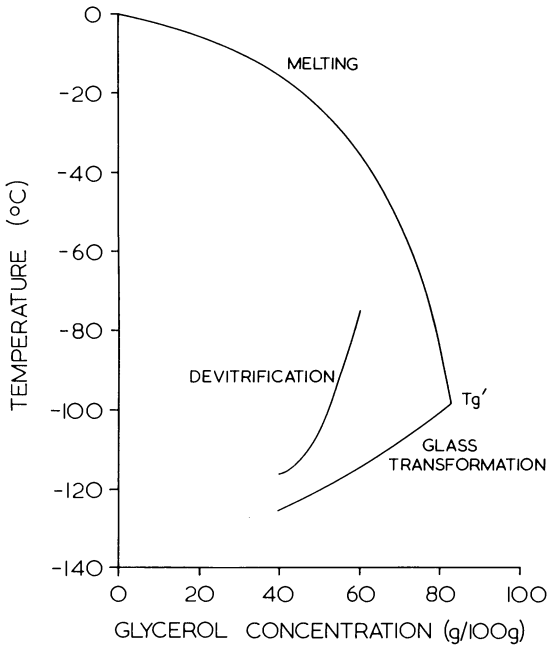

Figure 7. A supplemented phase diagram for glycerol/water. The intersection of the melting curve and the glass transformation curve at Tg′ indicates the minimum concentration of glycerol that will vitrify irrespective of cooling rate.

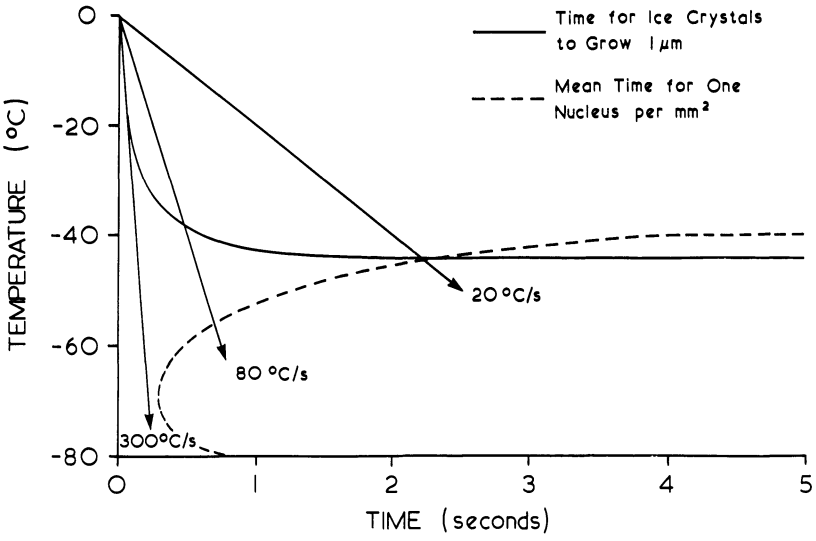

Figure 8. Diagram showing the time and temperature dependence of ice crystal growth and of ice nucleation in a thin film of 50% polyvinylpyrrolidone solution. The arrowed lines indicate cooling trajectories that should avoid nucleation, produce nucleation without ice, or produce ice. See text for futher discussion. (Reproduced with the permission of the Society for Experimental Biology.)

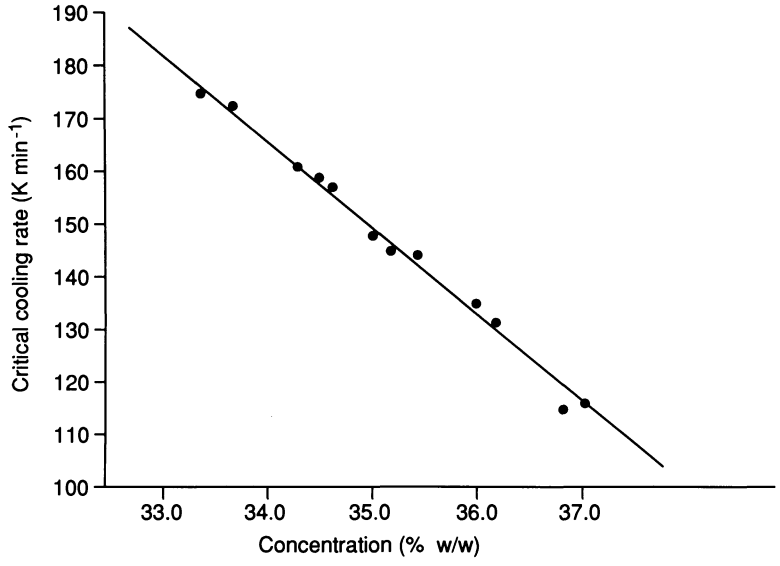

Figure 9. A plot of critical cooling rate to avoid crystallization of ice in solutions of propan-1,2-diol. The data was kindly provided by Dr. R.L. Sutton. (Reproduced by permission of Cryoletters.)

their paper [1], although the presence of extracellular ice would render the term "vitrification" inappropriate in today's usage. Had it been possible to reach the concentration of glycerol required to vitrify (Tg′ – see Figure 7) *before* cooling was initiated, true vitrification of the whole system would have occurred, but this would require c.80g% – far beyond the toxic limit at °C. There are, however more favourable solutes than glycerol, perhaps the most encouraging recent additions to the list being propan-1,2-diol and butan-2,3-diol [28] which will probably vitrify at a concentration of 50-55%.

This approach may be termed the "equilbrium approach" to vitrification: the system will vitrify no matter how slowly it is cooled. There is, however, another approach that is best illustrated by Figure 8: the first stage in the process of freezing is nucleation, and nucleation has an unusual temperature-dependence in that it becomes more active rather than less active with reduction of temperature, until it is limited by viscosity. However, the growth of ice crystals has a more usual temperature dependence – it is slowed and eventually arrested by cooling. The interesting point is the manner in which these two processes interact: the rate of ice crystal growth in the solution illustrated in Figure 8 has reached low values before the temperature zone for active nucleation is entered. Consequently three possibilities exist: if cooled sufficiently rapidly the sample may escape both nucleation and ice crystal growth; if cooling is more rapid the sample may be nucleated but without ice crystals; or, with slower cooling, the sample may nucleate and ice then form. In fact, it is not quite correct to superimpose cooling trajectories on the data of Figure 8 because that data was obtained under isothermal conditions [29], and this actually helps us – when such data is corrected for conditions of continuous cooling the permissible cooling rates are made slower. Data for propan-1,2-diol are shown in Figure 9 [29].

The data of Figure 8 carries another message for woud-be non equilibrium vitrifiers, and this concerns warming: as the temperature is raised the sample traverses the nucleation zone *first* and then the zone of ice-growth, so the stage is set for freezing to occur during warming even if it was avoided during cooling. The obvious remedy for this situation is ultra-rapid heating, and irradiation with microwaves is the obvious technique to use because of its potential for depositing energry uniformly as well as rapidly even in bulky samples. Considerable effort is now being devoted to the definition of the problem of microwave heating of appropriate systems from very low temperatures [30] and to its solution. Other manoeuvres that may help include the addition of materials that retard the growth of ice crystals, such as the so-called "anti-freeze" glycoproteins found in some fishes and insects [31], and the use of extremely high hydrostatic pressures to depress both the freezing and the nucleation temperature [32].

Conclusions

Much remains to be discovered concerning solutes that will permit vitrification at realisable rates of cooling, yet will also be compatible with viability. When this has been done it will be possible to study critical systems in the vitreous state: it should not be overlooked that no system that is succeptible to damage by extracellular ice has yet been successfully vitrified, and all those systems that have been preserved by vitrification (early embryos, monocytes, pancreatic islets [see 33]) can equally well be preserved by conventional freeze-preservation methods. The problems of vitrifying organized systems remain formidable, but it is towards vitrification that the signposts for preservation of these refractory systems clearly point.

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ICE NUCLEATION AND GROWTH IN VITRIFIABLE SOLUTIONS

R.J. Williams, P.Mehl, D.L. Carnahan

An important recent trend in cryobiological practice is the revitalization of Luyet's idea, over half a century old, that since ice crystallization may be lethal, the glassy or vitreous state is the only state compatible with cryopreservation [1]. Most living things require a liquid state in which certain physiological conditions are met. A glass is a special kind of liquid, one whose viscosity is so high that its fluidity is not apparent. Since chemical processes require movement of molecules, they will occur at much lower rates in a glass than in ordinary liquids. Luyet's original concept, then, was that life could be immobilized and preserved in a living state by changing the time scale on which metabolic and other biological processes occurred: by modifying the relaxation time constants of its liquids rather than altering its physical state. Luyet's original procedure, designed to exploit the time-expanding potential of both vitrification and low temperatures, was quench cooling to below the temperature at which the specimen's liquids become noncrystalline solids, the glass transition temperature. It was also mostly unsuccessful [2]. The new practitioners approach vitrification quite differently, depending upon better cryoprotectant formulations which allow more latitude in freezing and thawing rates, but the central difficulty persists: cells and tissues may survive in the liquid state at ambient temperatures, or in the vitreous state at very low temperatures, but must pass through a range of temperatures at which potentially damaging crystallization can occur going from one to the other. Retrieval requires a second passage, often the more hazardous one.

It has been acknowledged for many years that a large proportion of freezing injury may occur during warming rather than during cooling [3]. If the temperatures had been very low, in many cases this has been demonstrated to be the result of ice formation during warming in solutions which had vitrified and not fully frozen during cooling [4]. This decomposition of a supersaturated solution above its glass transition temperature into its crystallizable components, called "devitrification", is not unexpected, because solutions which vitrify are quite cold and concentrated. It has been assumed that ice nuclei do form during cooling but find themselves in an environment too viscous to permit detectable

growth. These nuclei would then be preserved in the glass formed at low temperature but would resume growth in the glassy melt when the solution becomes warm and fluid enough. When one cools a small sample of a dilute solution, it normally drops several degrees below its freezing point before it suddenly and thoroughly freezes. This is the inevitable result of the catalysis of ice by some foreign (heterogeneous) substance. Such nuclei form ubiquitously in materials not specially handled to exclude them. Though there may be many such heterogeneous nuclei in the sample, only the most efficient are seen [5]. This pattern changes in sufficiently concentrated soultions. Solutes which depress the melting point of a solution also depress the temperature at which nuclei become active. As a nucleus accretes ice, the solution around it concentrates and ice growth is impeded by an increasing viscosity. Ultimately, a concentration is reached at which the amount of ice formed is unmeasurable by standard methods.

In the absence of heterogeneous nuclei, pure water supercools about 40°C when homogeneous nucleation, ice formation by random fluctuations in water alone without catalysis from foreign materials, occurs. In their classic differential thermal analysis experiments on aqueous solutions of a number of solutes, Rasmussen and MacKenzie [6] systematically studied homogeneous nucleation and measured its suppression by solute concentration. They were able to separate homogeneous nucleation by dispersing their aqueous sample in hydrocarbon emulsions. Since their emulsions contained millions of isolated droplets, heterogeneous nuclei were confined to a small fraction [7]. Such nuclei did not interfere with the obsrvation of homogeneous nucleation in droplets not containing heterogeneous nuclei. Most importantly for cryobiology, there is a concentration limit above which ice forms during warming but not during cooling. For sucrose, this concentration is 62%. Recently, Charoenrein and Reid [8] examined emulsified samples sucrose solutions up to 50% containing large numbers of heterogeneous nuclei in a scanning calorimeter. Their ice nucleating agents (INA's) were *Pseudomonas* spp. bacteria, the most efficient heterogeneous nuclei then known. Their data demonstrate that homogeneous nucleation is also suppressed by solutes, though only by about two-thirds as much as homogeneous nucleation.

The experiments we report investigate the devitrification of sucrose solutions between 62% (w/w), above which no water freezes during cooling, and 80%, above which there is no freezeable water. Our data indicate that ice nucleated either heterogeneously or homogeneously in these solutions too concentrated to freeze during cooling is nonetheless directly relatable to the nucleation and ice growth during cooling studied at lower concentrations by Charoenrein and Reid. In addition, we report a third source of nucleation which can occur only below the glass transition, nucleation at interfaces, and especially those formed by fracturing. We will describe factors influencing the growth of ice after it has

been nucleated. Finally, we will describe procedures for minimizing ice damage in cryopreserved specimens.

Methods and materials

Two instruments were used in the experiments reported in this paper. The first was a differential scanning calorimeter (Perkin-Elmer DSC-4, Norwalk, CT USA) which we have adapted to operate reliably at low temperatures [9]. Samples varied from 3 to 25 mg and were sealed into sample pans originally designed for robotics use (BO14-3003/-3015 or BO143-004/-021) which offer over ten times the sensitivity of standard pans. For scanning experiments, the TADS computer program was used, and all experiments were performed at 20°C/min. This is a compromise which allows sensitivity in the display of glass transitions without seriously distorting the temperatures at which they are observed. For isothermal measurements, the DSCI program was used. Samples were cooled to –100°C at 20°C/min, warmed to the temperature desired at 40°C/min where it was held until crystallization was essentially complete. To correct for instrumental systematic error, the sample was kept at the "limiting" glass transition temperature to complete crystallization, cooled, scanned again in an identical manner and the difference between the two files recorded and analyzed.

The second instrument was a cryomicroscope, assembled from an interopherometric microscope with long working distance objectives (Zeiss INTERPHAKO, Jena, DDR) and a temperature-programmable stage capable of being cooled below –150°C (Linkam THM-600, Surrey, UK). The microscopic objective lens most used was a 16/0.2 planapochromat. The size of the sample placed between two 16 mm round coverslips on the cooling stage varied from 5 to 15 µl. For routine cryomicroscopy and photomicrography, the temperature was increased or decreased at either 10°C or 20°C/min and stopped arbitrarily. Solutions were mixed by weight in scintillation vials and dissolved at 65°C or if necessary 95°C. Concentrations were verified with an Abbe' refractometer (Bausch and Lomb). Some solutions contained additionally up to 5% of materials reported to possess unusual properties related to the stability of biological glasses; viz., putrescine, spermidine, boric acid, hexanoic (caproic) acid, and freeze-dried INA bacteria (Snomax Technologies, Rochester, NY, USA).

Results

Ice nucleation

Figure 1 shows thermograms of 65% sucrose solutions in which no heterogeneous nuclei were present. Sealed samples were cooled to –40°C, –60°C, –80°C, etc. Scanning thermograms were made of the warming

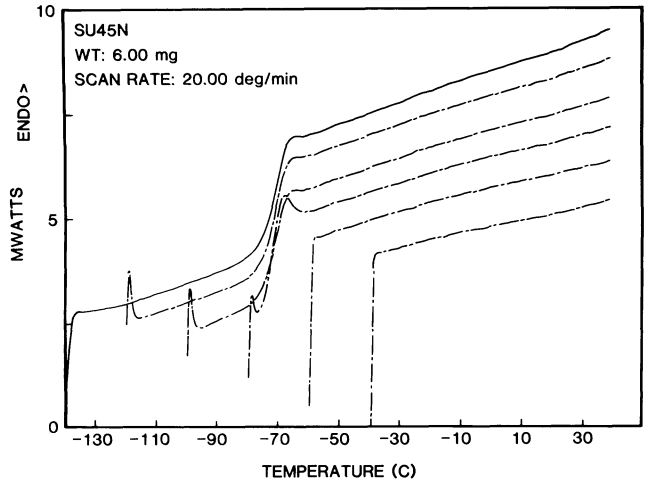


Figure 1. DSC thermograms of a 65% (w/w) sucrose solution from which heterogeneous nuclei have been excluded, cooled at 20°C/min to –40°C, –60°C, –80°C, etc. and rewarmed. Except for the startup artefact, and the glass transition in samples cooled below –80°C, there is no evidence for thermal activity in this sample. The sample had been covered with 2-methyl butane to prevent airborne nuclei from migrating to its surface.

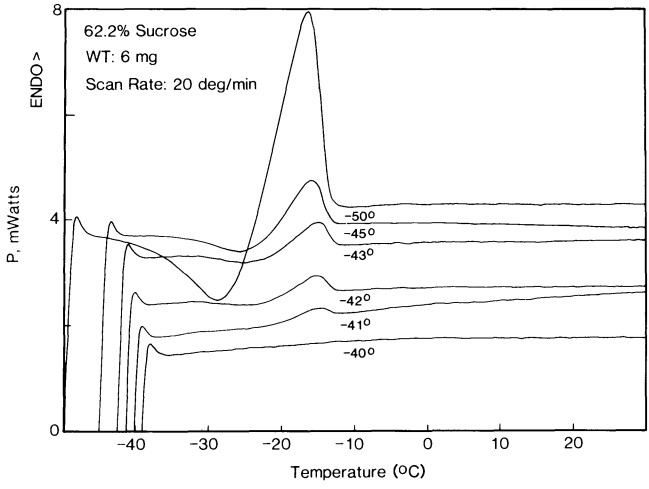


Figure 2. DSC thermograms of a 62% sucrose solution containing INA's (see text). The sample was cooled at 20°C/min to –40°C, –41°C, –42°C, etc. and rewarmed. The sample warmed from –40°C shows no events other than the startup artefact; all samples warmed from lower temperatures show an endotherm at about –15°C, indicative of melting. This is evidence that ice was nucleated between –40°C and –41°C.

process. The 4 μl of sucrose solution had been covered with 6 μl of 2-methyl butane (isopentane). Samples cooled to –40°C and –60°C show a starting transient (vertical rise) but after the calorimeter has equilibrated, the thermogram is eventless. Thermograms made from samples cooled to –80°C and below show in addition to this transient a sharp rise in heat capacity beginning at about –76°C. This is the glass transition, and corresponds well to values reported by MacKenzie and Rasmussen [10]. Above the glass transition, no thermal events are seen. The view of such a specimen in the cryomicroscope is of a clear window whose glass transition is invisible in brightfield illumination and marginally discernable by interferometric methods. We interpret this as evidence that no ice has developed in this specimen even after it had been cooled to –140°C.

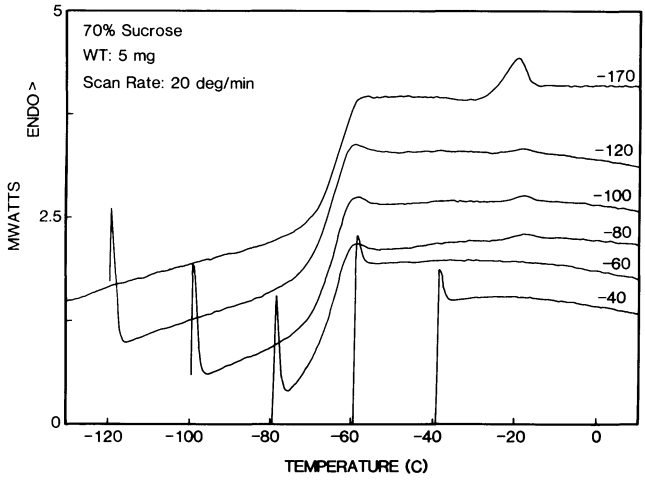


Figure 3. DSC thermograms of a 70% sucrose solution cooled at 20°C/min to low temperatures and rewarmed. Samples rewarmed from –40°C and –60°C show no thermal events. Samples cooled to between –80°C and –120°C show a small thawing event, accounting for less than 1% of the freezeable water in the sample. This form of nucleation occurred at or below the glass transition temperature. Since this sample was not covered with 2-methyl butane, it probably resulted from the migration of nuclei in the vapor space onto the surface of the sample. In contrast, the sample cooled to –170°C showed a large endotherm in which half of the freezeable water melted. For explanation, refer to Figure 5 and text.

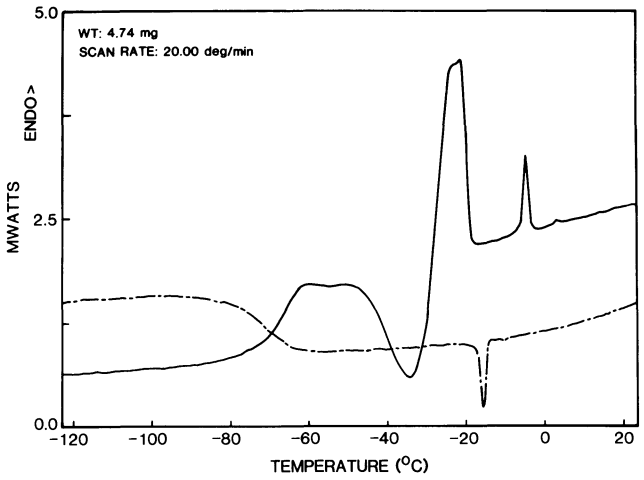


Figure 4. DSC thermograms of a 68% sucrose solution containing 5% hexanoic acid. During cooling (dashed curve) a small exotherm (freeze) is seen at above –20°C, and a glass transition at about –70°C. During warming, a symmetrical glass transition is seen, followed by an exotherm representing the freezing of water during warming (devitrification). This is followed by two endothermic melts, indicative of a liquid-liquid phase separation. See text and Figure 5 for additional details.

The family of curves in Figure 2 show the effect of INA bacteria when present in a sample of 62% sucrose. The warming from –40°C resembles that in Figure 1. However, samples warmed from –41°C, –42°C, –43°C, etc. do show a progressively increasing series of endotherms and at –50°C, a pronounced exotherm followed by a large endotherm. These represent ice formation during warming of the glassy melt, or devitrification, followed immediately by the melting of this ice at the melting point, below –15°C. We interpret this as evidence that the INA's had nucleated ice between –40°C and –41°C.

When samples not covered with isopentane and without added INA's were cooled and heated in the DSC, the results seen in Figure 3 were obtained. Warming from –40°C and –60°C produced eventless records. Warming from –80°C to –140°C produced in addition to the glass transition a small melting endotherm, indicating the freezing of less than one percent of the freezeable water. This probably resulted from deposition of nuclei formed in the vapor space on the unprotected sample surface. Cooling the sample to –170°C or below (solid line, top) produced a melting endotherm representing half or more of the freezeable water in the sample. This represented formation of nuclei well below the glass transition temperature.

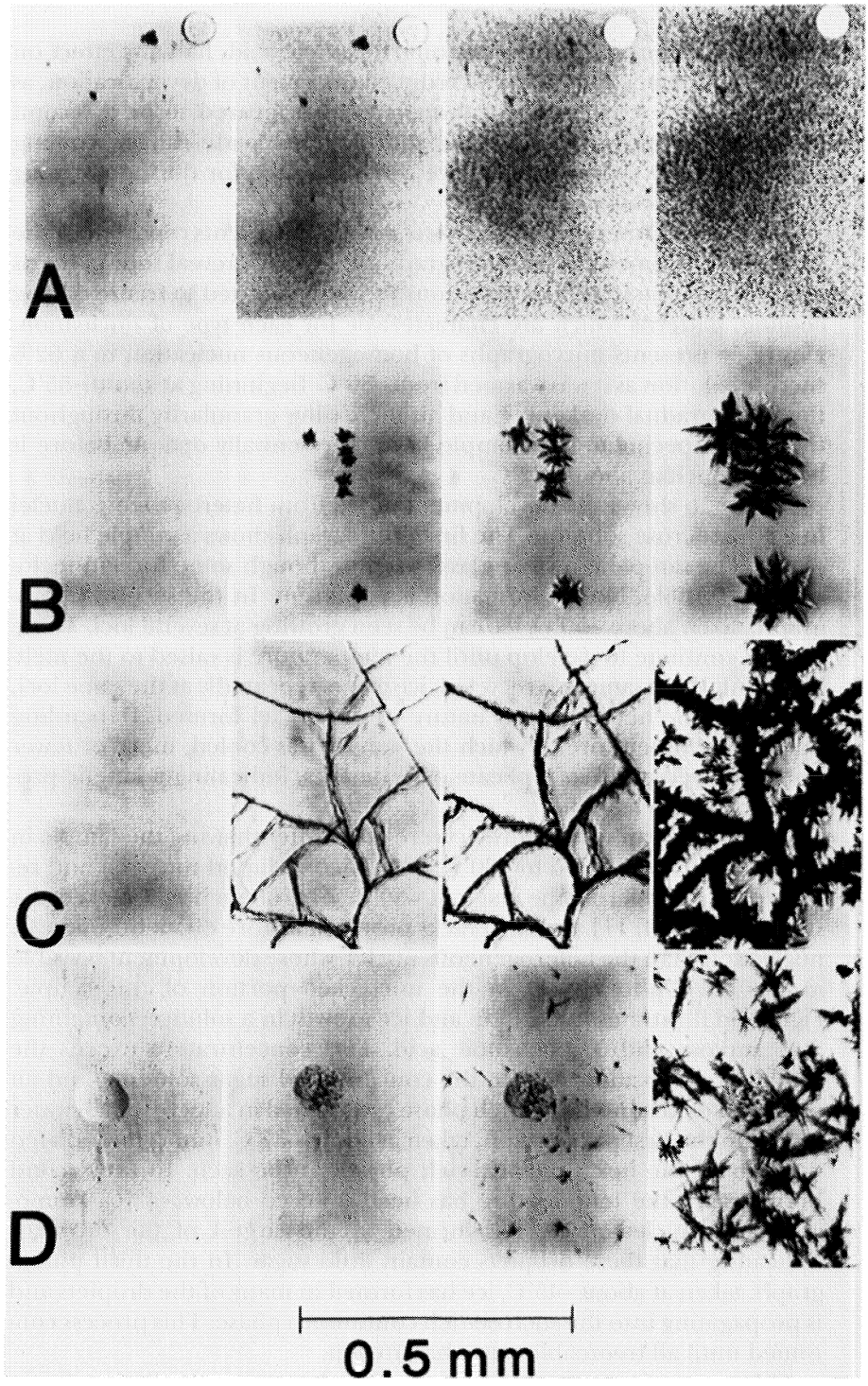
Of the organic additives, only one, hexanoic acid, had any effect on the thermograms. This material reduced the extent of devitrification, as seen in Figure 4 (solid line), though there appeared to be a second minor melt. Examination of the thermogram made during cooling (dashed line) revealed that the ice not accounted for during warming had formed during cooling.

The cryomicroscope allowed us to sort out the events reported by the scanning calorimeter. The micrographs in Figure 5 reveal four patterns of devitrification in sucrose solutions too concentrated to freeze during cooling, patterns which are characteristic for each type of nucleation. Figure 5a presents micrographs of homogeneous nucleation in a 62% sucrose solution as it was warmed from –80°C. Beginning at about –55°C, there is a gradual darkening and an increasing granularity throughout the whole specimen. The sample became essentially opaque before it began to melt at about –22°C.

Figure 5b shows the development of ice from heterogeneous nuclei in a 65% sucrose solution. The first photograph shows a sample held at –80°C. The sample is a clear glassy window though some foreign inclusions are visible. No ice formation is apparrent. In the second photograph, taken above –50°C, ice can be seen growing at several loci. These crystals continue to develop until the temperature is raised to the melting point. If the sample is recycled, ice grows repeatedly at the same loci, indicating the heterogenous nature of the nuclei formed. Depending upon the temperature to which the sample was cooled, more or fewer nuclei are recruited in replicate experiments, indicating a kinetic process for this nucleation.

The photographs in Figure 5c were taken after thawing the sample in Figure 5b. It was cooled to –80°C, quenched in liquid nitrogen and replaced in the microscope stage at –80°C. The quenching cracked the sucrose glass [cf. 11] and the cracks provided a more efficient source of nucleation than the heterogeneous nuclei, whose development is visible in the third photograph in the uncracked portion of the sample. Figure 5d illustrates nucleation and ice growth in a solution containing 56% sucrose and 5% hexanoic acid. This concentration exeeds the solubility of hexanoic acid in the concentrated sugar solution, and an emulsion of hexanoic acid-rich phase has formed in a sucrose-rich water phase. In the first photograph, taken at about –37°C, numerous isolated droplets of the hexanoic acid-rich phase can be seen. In the second photograph, the temperature has been lowered below –43°C. Homogeneously nucleated ice has formed in the largest of the droplets, indicating that these droplets contain little sugar. In the third photograph, taken at about –45°C, ice has formed in many of the droplets and is propagating into the sucrose-rich continuous phase. This process continued until all freezeable water had frozen.

Other experiments were performed to determine the limits of concentration that would permit heterogeneous or homogeneous nuclea-


A
B
C
D
0.5 mm

tion. A 74% sucrose solution containing INA would form ice if cooled to –60°C or below. Without INA, it never froze unless cracked by quenching. A 65% solution containing INA formed heterogeneous nuclei at about –45%, but without INA did not freeze homogeneously. A 62% solution seeded with INA bacteria showed an interesting behaviour. Cooled to –40°C or below, it grew ice from heterogeneous nuclei. Cooled to –80°C or below, it froze homogeneously, and this more evenly distributed ice formation precluded the development of the heterogeneous nuclei.

Ice growth

The rate at which an ice crystal grows during the devitrification process is most conveniently studied at a constant temperature. A series of thermograms showing devitrification in a 62% sucrose solution is shown in Figure 6. The sample held at –77°C is eventless; samples held at progressively higher temperatures show exotherms whose intensity increases and whose time course shortens. To analyze the kinetics of crystallization, the most traditional model is the Johnson-Avrami equation:

$$X = 1 - \exp\{-[K(T)t]^n\}$$

where:
X = the fraction crystallized; $K(T) = K_0 \exp(-E^*/RT)$; T = temperature (^{0}K), t = time; E^* = the activation energy; K_0 = a constant dependent upon the density of nuclei; and n = the Avrami constant.

The details have been reviewed by Christian [12]. It can be shown that when one plots the time at which the maximum rate of crystallization occurs as a function of 1000/T (Figure 7), one obtains a slope proportional to E^*. A plot of $\log[-\log(1 - X)]$ as a function of time of exposure, t, (Figure 8) gives lines whose slopes are equal to the Avrami exponent. By these methods, E^* is found to be 120 kJ/mol. The value of n is tem-

*Figure 5.*Cryomicrographs of devitrification in sucrose solutions.
A. Homogeneous nucleation. Sample has been cooled to –80°C and rewarmed. Nuclei are uniformly present and ice recrystallizes into progressively larger crystals.
B. Heerogeneous nucleation. Sample cooled to –80°C and rewarmed. Ice grows repeatably from the same loci.
C. Fracture nucleation. Sample in 5B cooled to –80°C, dipped into liquid nitrogen and reinserted in the cryomicroscope at –80°C before warming. Ice grows aggressively from fracture interfaces, compared to isolated heterogeneous nuclei, visible in the third micrograph.
D. Nueation in a two-phase system. The droplets of hexanoic acid-rich phase can be seen at –37°C. These have homogeneously nucleated at –42°C, and subsequently spread into the surrounding sucrose-rich phase.

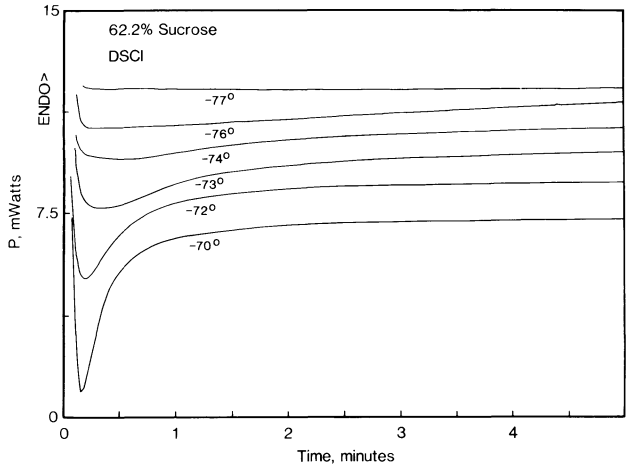


Figure 6. "Isothermal" thermograms of 62% sucrose solutions illustrating the rate of ice growth as a function of temperature. These thermograms have been corrected for instrumental systematic error. Sample was cooled to –100°C at 20°C/min, rewarmed to the temperature stated for each thermogram and held at this temperature. The rate and extent of the development of the exotherm is indicative of the growth of ice. From such data, the parameters of the Johnson-Avrami equation can be calculated.

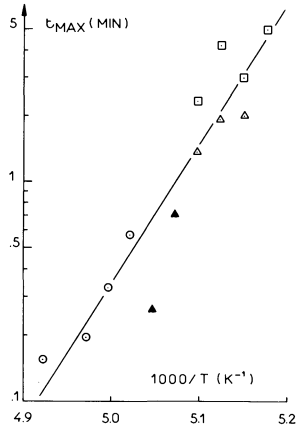


Figure 7. Plot for 62 wt% sucrose for the time in minutes, t_{max}, corresponding to the occurrence of maximum crystrallization rate as a function of 1000/T, where T is the temperature at which the isothermal crystallization is recorded by differential calorimetry. Different samples are represented by different symbols. The solid line is a least squares fit with a correlation coefficient of 0.935 and an estimated E* of 120 kJ/mol.

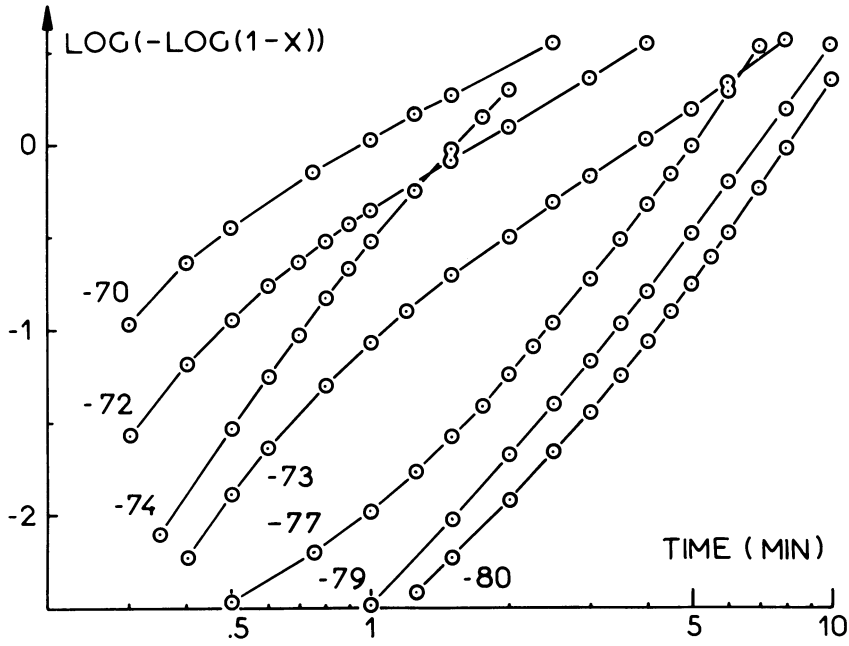


Figure 8. Johnson-Avrami plot of the isothermal log[–log (1-X)], where X is the ice crystallization fraction, as a function of time of exposure at a series of temperatures. The Avrami coefficient, n, is estimated as varying with temperature between 0.6 and 1.54.

perature dependent, varying from 0.6 to 1.54. The critical warming rate, the rate at which the sample must be warmed in order to eliminate devitrification, is calculated to be 10^6°C/sec.

Discussion

The Johnson-Avrami equation is exponential in time and doubly exponential in temperature. Interpreted literally, it implies that once ice nuclei are present growth will proceed inexorably. This classical model has already been applied to different cryoprotectant solutions [13,14]. The values of the constants depend upon the nature of the solute, the hydrogen bonds formed with water and the concentration. Their values reflect considerable differences in the abilities of solutions to avoid crystallization during cooling and warming. It can be seen from the results that sucrose would be an impracticable cryoprotectant on these grounds. In contrast, a systematic investigation of the stability of aqueous solutions of low molecular weight mono- or polyalcohols shows that devitrification can be avoided in relatively concentrated solutions by using warming rates of about 200°C/min [15,16].

The first alternative to restricting devitrification is to limit ice growth. Fahy [17] has elected to use high warming rates in his experiments with

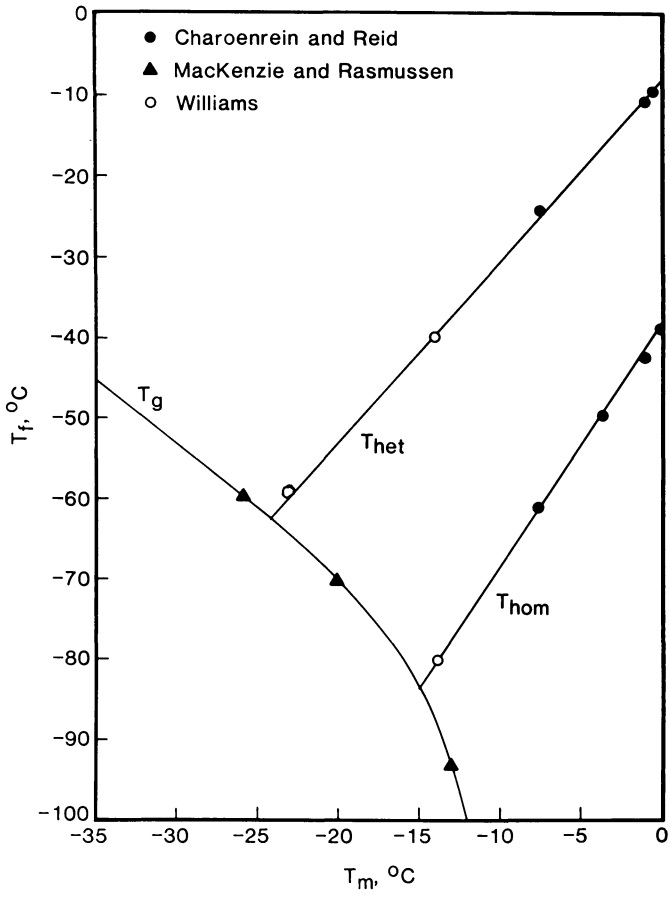


Figure 9. The freezing point of a sucrose solution (either during cooling or by devitrification during warming) as a function of its melting point. The presence of heterogeneous nuclei (INA bacteria) greatly elevates the temperature at which ice nuclei form, and greatly increases the concentration required to suppress nucleation. Nuclei cannot form by these mechanisms below the glass transition, though they may form during the fracturing process.

vitrification of kidneys. The principal obstacle that they have encountered is reconciling the need for high concentrations with the problems of toxicity.

The other alternative is to avoid or to reduce the occurrence of nucleation. In Figure 9, we have mapped the conditions under which nuclei can form. It extends the diagram presented by Charoenrein and

Reid [8] to include our calorimetric data and has numerous implications for cryobiology. The temperatures at which nuclei develop are presented as a function of the equilibrium melting temperature. INA bacteria are able to form ice at higher temperatures and concentrations than heterogeneous nuclei, and in both instances our results and the published data agree well. Both heterogeneous and homogeneous nucleation end abruptly at the glass transition, here redrawn from MacKenzie and Rasmussen's data [10]. Nucleation of ice at fracture faces can occur at temperatures below the glass transition temperature when the material has become brittle enough to fracture. This temperature is not as well defined as the other limits, and is even more highly dependent upon rates of cooling than are the other nucleation events [7].

All cryoprotectants in common use are highly soluble in if not actually miscible with water. Nonetheless, the samples in Figures 4 and 5d of sucrose solutions containing a second immiscible phase offer an important metaphor. The nuclei that formed ice in this dilute second phase were homogeneous with respect to their own phase, but heterogeneous from the perspective of the concentrated sugar-rich phase surrounding them, a phase that they were quite efficient at devitrifying. Tissues cryoprotected by a non-penetrating agent such as hydroxyethyl starch, or even tissues not completely equilibrated with the nomically permeating cryoprotective agents such as dimethylsulphoxide or the polyols, will contain linging cells which are in effect a second phase nearly as dilute as the hexanoic acid-rich phase was in our experiments. Due to this lower solute concentration, ice crystallization would occur well above the glass transition temperature and disseminate ice throughout the sample.

One can from the above begin to devise a rational method for ameliorating the damaging effects of devitrification. Rasmussen has proposed [18] that homogeneous nucleation is a spinodal decomposition and that its occurrence at equilibrium at some temperature is an inevitability. He and MacKenzie long ago demonstrated that this temperature is a function of freezing point depression and nearly independent of chemical nature of the solutes [6]. Thus, the prevention of heterogeneous nuclei is not a factor which can be significantly manipulated in the design of cryoprotective solutions. The obvious strategy from Figure 9 is to increase solute concentration to the extent that homogeneous nucleation will not have occurred when the material vitrifies. Again, sufficient concentrations, though lower than those required to suppress heterogeneous nucleation, may be injurious.

The INA bacteria, which have been a boon to cryobiologists wishing to restrict supercooling, are proving a distinct impediment to those wishing to vitrify their material. Bacteria accidentally released into our laboratory persist, becoming less and less efficient, with a half-life of two or more weeks. Fortunately, they and other heterogeneous nuclei can also be effectively removed by routine sterile techniques: steam autoclaving, 0.2 μm

filtration, sample preparation in a laminar flow hood and cleaning of the sample pans with an alcohol-ammonia glass cleaner such as "Windex". There is little doubt that heterogeneous nucleation can be largely eliminated as an impediment to long term storage by vitrification.

Of all the variables, the glass transition temperature is the most amenable to manipulation. A glass transition can be considered a bulk phenomenon. Solutes vary widely in the extent to which the plasticizer, water, depresses their glass transition temperatures [19] but with a homologous series of compounds, the glass transition temperature rises with the degree of polymerization. This is an important consideration for the design of cryoprotective solutions because as the degree of polymerization rises, the melting point temperature depression per unit concentration decreases, which in turn decreases the amount of osmotic stress imposed per weight percent of solute. As an example, the glass transition temperature and the concentration at which it was reached proved to be the critical determinants of survival in human monocytes cryoprotected with hydroxyethyl starch [20]. At 20% the hydroxyethyl starch they used imposes a trivial osmotic stress on the cells. When enough water has frozen out to form the "limiting" glass at –20°C, the HES has been concentrated to 70%, restricting the osmotic stress to only 2.5 times isotonic. This is within the transient osmotic tolerance of these cells and above the temperature at which intracellular nuclei form.

Fracturing in aqueous solutions is liable to occur when the sample is cooled below the glass transition temperature. From the viewpoint of cryobiology, it is preventable. Plasticizers may be of some benefit. The traditional practice of immersing specimens in liquid nitrogen or its vapor, producing rapid temperature excursions, exposes the sample to the hazard of fracturing in a manner that is difficult to control. Fractures through a vitrified suspension of red cells will only destroy a few cells. Fractures through an organ, such as the frog hearts vitrified by Rapatz [21] will also destroy only a few cells, but will destroy the organ. Even surface fractures have recently been reported in human heart valves lowered from nitrogen vapor into the liquid nitrogen in storage vessels [22]. And even if it can be controlled to the extent that tissues or organs are not physically injured, fracturing will still provide an invasive surface for the propagation of the second most efficient form of ice nuclei we have observed.

Concerning the usual cyroprotectant solutions for organ vitrification, fracturing is problematical partly because of the equipment commercially available for the storage, which at the moment offers two alternatives, mechanical refrigerators or liquid nitrogen. If nothing else, Figure 4 should convince us that storage at –80°C in a two-stage mechanical refrigerator is inadequate. It is cold enough to produce nuclei, but not cold enough to vitrify. Ice would not grow as rapidly as it does at the higher temperatures where we watch it develop in seconds, but it would grow relentlessly in hours, months or years to produce injury.

Living cells and tissues are far more complex and interesting than the little droplets of sugar water described above and, independently of the suspending media, they have a set of glass transitions of their own, at least three [23]. Nevertheless, the lessons we have learned from simple binary solutions should be a good beginning for devising means for minimizing devitrification injury in materials of medical interest. cryoprotectants must restrict nucleation and limit ice growth to values which can be overcome by reasonable rewarming rates.

Acknowledgements

We thank Harold T. Meryman, MD, for his support, encouragement and criticism.

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LIPOSOMES AS A MODEL SYSTEM FOR THE CRYOPRESERVATION OF PERIPHERAL BLOOD CELLS

H. Talsma, M.J. v. Steenbergen, P.J.M. Salemink, D.J.A. Crommelin

Liposomes can be looked upon as synthetic cells, as they are closed vesicles consisting of phospholipid bilayers which surround an internal aqueous volume. Therefore, they might mimic the behaviour of peripheral blood cells during cryopreservation in several aspects. Freezing and freeze-drying have been proposed as stabilization techniques for the long-term storage of peripheral blood cells. However, attempts to store these cells in freeze dried form with complete retention of contents and activity have not been very successful until now. Major problems encountered were leakage of cell contents and alterations in cell size. A number of potential damaging effects during cooling of cell dispersions have been suggested, including crystallization of the cryoprotectant, crystallization of internal ice and osmotic forces originating from freeze concentration. The objective of this study was to investigate the behaviour of negatively charged liposomes in a dispersion containing cryoprotectant during a freezing/thawing cycle. Freezing and melting processes were monitored by differential scanning (DSC). The extent of marker (5 mM carboxyfluorescein) encapsulation was measured before and after the cycle.

The liposomes were prepared according to the "film" method and extruded through 0.6 μm polycarbonate filters to obtain narrow particle size distributions. The bilayers of the vesicles were composed of hydrogenated soybean phosphatidylcholine and dietylphosphate (molar ratio 10/1). The hydration solutions contained 10 mM Tris buffer (pH=7.4) and cryoprotectant ((glycerol, propylene glycol, mannitol).

In Figure 1 the DSC cooling curves of a Tris-buffer containing mannitol, a liposome dispersion in Tris-buffer (section A) and a liposome dispersion in Tris-buffer containing mannitol (section B) are shown.

The first cooling peak represents the crystallization of "bulk" water, the second peak the crystallization of maximally supercooled water at the homogeneous nucleation temperature. The liposome dispersion containing mannitol showed an increased water crystallization enthalpy starting at –45°C compared to the lipid dispersion without cryoprotectant, indicating that part of the water in the dispersion remains

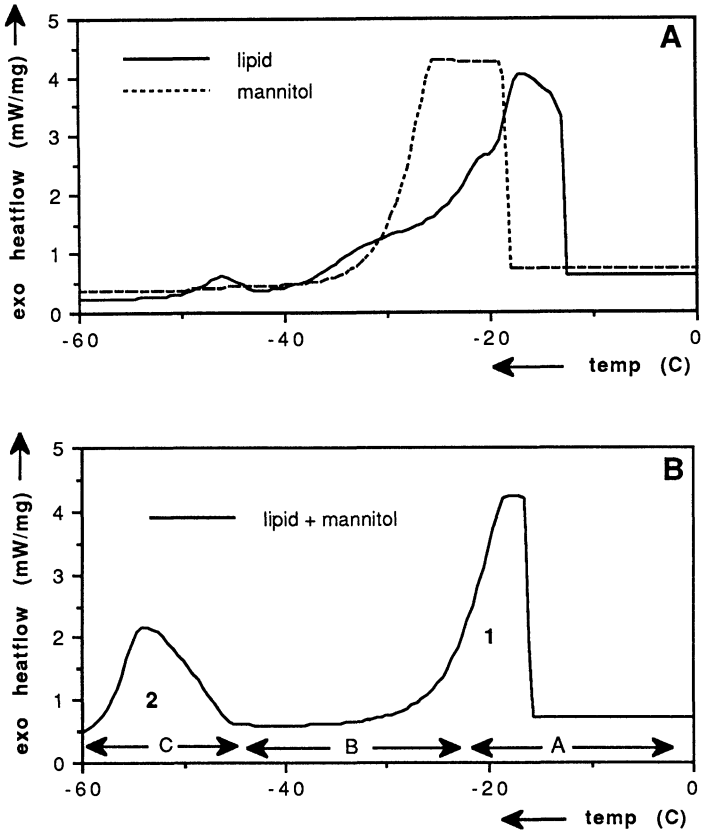

Figure 1. DSC cooling curves. A: 80μmol lipid/ml liposome dispersion in 10 mM Tris pH=7.4; 11.2% mannitol in 10 mM Tris pH=7.4. B: 80 μmol lipid/ml liposome dispersion in 10 mM Tris pH=7.4 containing 11.2% mannitol. Cooling rate 10°C/min.

Table 1. Crystallization onset temperature of peak 2 for different cryoprotectants. $T_{e.o.}$ = extrapolated onset temperature of the peak. Lipid composition: Phospholipon 100H/dietylphosphate 10:1, multilamellar vesicles in 10 mM Tris pH=7.4. Cooling rate 10°C/min.

Cryoprotectant	**% (w/v)**	**$T_{e.o.}$ (°C)**
Mannitol	11.2	–45.1
Glucose	30	–52.7
Glycerol	30	–61.3
Propylene glycol	30	–76.1

supercooled under these conditions until the homogeneous nucleation temperature has been reached. The presence of cryoprotectant generally increases the amount of supercooled water, as shown by the enhanced crystallization enthalpy at low temperature (Figure 1B).

The homogeneous nucleation temperature depends on the concentration and type of cryoprotectant used as shown in Table 1. The results generally agree with those published by MacKenzie [1].

In Table 2 the crystallization enthalpy of peak 2, of the liposome dispersion containing mannitol, after different isothermal storage times at –25°C is shown.

After the crystallization of "bulk" water part of the uncrystallized water in the dispersion slowly converts into ice during storage as long as the homogeneous nucleation temperature has not been reached.

When monitoring the loss of encapsulated marker, three regions can be distinguished as indicated in Figure 1B.

A. Cooling the dispersion to a temperature just below the heterogeneous nucleation temperature and within 30 seconds followed by reheating (with 50°C/min) up to 20°C does not result in substantial marker loss (1-3%).
B. Cooling the dispersion to temperatures between the heterogeneous and homogeneous nucleation temperature and within 30 seconds reheating up to 20°C resulted in a cooling time and temperature dependent marker loss.
C. Cooling the dispersion to a temperature below the homogeneous nucleation temperature and within 30 seconds reheating up to 20°C resulted in complete marker loss.

It was observed that the degree of marker leakage increases, whereas the crystallization enthalpy (peak 2) decreases with an increasing duration of the cooling step.

As a possible explanation for these results, it is suggested that after crystallization of the "bulk" water in the dispersion the liposomes are captured in a slowly concentrating cryoprotectant solution between the

Table 2. Crystallization enthalpy of peak 2 after different isothermal storage times at –25°C. Lipid composition: Phospholipon 100H/dietylphosphate 10:1, multilamellar vesicles in 11.2% mannitol/10 mM Tris pH=7.4. Cooling rate 5°C/min.

Isothermal time (min)	**Crystallization enthalpy (J/g)**
4	6.8
9	3.0
18	1.5

growing ice crystals. These concentrating cryoprotectant solutions induce a growing osmotic pressure difference over the lipid bilayer during cooling. This osmotic pressure difference is compensated by water transport through the bilayer. During thawing the water transport through the bilayer is not fast enough to avoid a, by osmotic pressure differences induced, burst effect. This burst effect is resulting in loss of internal material, immediately followed by resealing of the bilayer (in literature described as "popping").

In conclusion it is possible to detect supercooled water in liposome dispersions using DSC. Supercooled water seems to play an important role in the stability of dispersions with respect to leakage of water-soluble markers. The lack of success of attempts to store liposome dispersions at subzero temperatures might be related to damage to the liposome dispersion caused by complete crystallization of the supercooled water or to osmotic forces during thawing.

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DISCUSSION

H.T. Meryman, P.C. Das

J.C. Bakker (Amsterdam, NL): Dr. Armitage, you made a general statement that below –130°C for all enzymes the activity is zero. Is it true for all enzymes and on which types of enzymes is that statement based?

W.J. Armitage (Bristol, UK): If you are going down to below –130°C, you are close to or below the glass transition of water and there will be no liquid water present. There will also be insufficient thermal energy to drive any chemical reaction and for these two reasons there should be no enzyme activity. At –80°C, on the other hand, there is still some liquid water present. Platelets cyropreserved at this temperature with glycerol, do not show some degradation of function with time, whereas platelets cryopreserved with DMSO do not show the same sort of degradation.

J.T. Derksen (Pasadena, CA, USA): Dr. Salemink, have you ever attempted to lyophilize cells?

P.J.M. Salemink (Oss, NL): We did attempt to lyophilize synthetic cells. Our results were only partially successful, but I am aware of papers in the literature published in 1985[1] and 1986[2] in which the authors have claimed 100% retention of soluble markers present within liposomes after freeze-drying and rehydration after chosing the right cryoprotectants. In this paper cyroprotectant was present in the extracellular as well as in intracellular medium and their specified concentrations resulted in full retention of the compounds after freeze-drying and rehydration.

1) Crowe LM, Crowe JH, Rudolph A, Womersly C, Appel L. Arch Biochem Biophys 1985;242:240-7.
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H.T. Meryman (Rockville, MD, USA): Dr. Salemink, I have a concern. You showed that the shelf temperature was maintained constant while the sample temperature rose progressively. The reason the sample temperature rises is because there is an increasing resistance to vapour flow and a reduced amount of heat or sublimation is being consumed at the drying boundary. Now, a most important factor in freeze-drying is that the sample temperature should never rise above some particular temperature, usually the eutectic temperature. Is it not a little dangerous to indicate a constant shelf temperature as a classic freeze-drying mode. When the sample temperature is too low, the drying is inefficient. If the sample temperature is too high, you are damaging the sample. In an ideal freeze-drying situation you might have the shelf temperature decreasing in the course of drying. Would you comment on that please.

P.J.M. Salemink: In this particular example the product could tolerate a whole range of freezing temperatures. So, this product was not very labile and we did not have to have the product temperature controlled within very strictly specified limits.

H.T. Meryman: I guess my concern is that your information might be interpreted as a recommended generic freeze-drying procedure. Many of the freeze-drying cycles that I have seen were ineffective because it was assumed that a constant shelf temperature or even an increasing shelf temperature during drying was the way to proceed, when in fact it may be exactly the opposite.

J. Over (Amsterdam, NL): Dr. McIntosh or Dr. Salemink could you give more detail about the influence of the freezing rate of a plasma or protein product for optimal freeze-drying. Our own experience has been that applying too high a freezing rate impairs sublimation, thus causing longer freeze-drying times. This has to do, of course, with crystallization of the ice and forming capillaries through which the water can evaporate and be removed from the bottle. But could you also say something about the influence on solubility of the final product.

P.J.M. Salemink: You mean the relationship between freezing rate of a plasma protein preparation and the solubility of the product.

R.V. McIntosh (Edinburgh, UK): You may remember I showed the different crystal structures in the freeze-dried vials of factor VIII concentrate. When the product was frozen on a –50°C cold shelf, we got these three types of structure: One which has a heterogeneous structure, having large crystals and a crust on the top, a second one which has a mixture of large crystals and a fine homogeneous structure, and the last one, which looks completely amorphous with a fine crystalline structure. In terms of solubility, following heat treatment this last type always dissolved

the best and showed the highest factor VIII recovery. So the question for us was how to obtain this structure in all of the vials.

J. Over: There is one other aspect that is of importance in this respect, the moisture content. So are you sure that the moisture content in all these bottles were the same?
R.V. McIntosh: Yes, they were the same. But the thing is that in a heterogeneous structure you may find different moisture contents in different parts of the plug.

G.M. Fahy (Rockville, MD, USA): Dr. Talsma, I wanted to know if the T_h that you observed in your liposomes went down when you did those experiments at –25°C and when you held for different intervals before cooling further.

H. Talsma (Utrecht, NL): The T_h was at the same temperature. The interval range is 4-18 minutes. I always found –45°C for the mannitol dispersions independent from the cooling conditions. Of course, for other cryoprotectants there is the possibility to reach lower temperatures before you reach the homogeneous nucleation temperature. But the T_h is independent from the cooling cycle, while the related heat-flow at the T_h is clearly time dependent.

G.M. Fahy: Dr. Pegg, as far as the vitrifiability of solutions is concerned, I disagree that T_g' is really the temperature that defines the required concentration. The concentration corresponding to T_g' is probably not actually reached in the real world, at least not in a glycerol-water system. You tend to reach a limiting concentration much sooner than that and proceed on downward to the glass transformation temperature without a further change in concentration. So, I think that $C(T_{g\ 1/2})$, is a nice ideal point on a phase diagram, but I do not believe that you get there during normal cooling.

D.E. Pegg (Cambridge, UK): I think in practice that is correct, but the physics is the physics. What I am talking about is the equilibrium at infinitely slow cooling conditions. You are talking about finite cooling rates and of course that does materially alter the situation. If I had judged my talk a little better, I would have got on to the point, that there are really essentially two approaches to achieving vitrification. One is an equilibrium approach, where you are not concerned with rates either of cooling or warming, where you are always to the right of the liquidus line and no matter what the rates of change in temperature are there will be no ice crystals formed. The problem with that appraoch is that you need such very high concentrations of cryoprotectant. I will agree that glycerol is not the one you would use; there are more favourable cyroprotectants and mixtures such as you developed. But this approach is not ruled out

completely. Mike Taylor[1] in my group has been looking at the maximum concentration of DMSO that rabbit cornea will tolerate: He is now up to 4.25 molar, and that is not the limit. So it may be that one can get very close to the equilibrium approach. If you have to use dynamic approach, you will have to cool more rapidly and have controlled rapid warming.

1) Taylor MJ. Cryobiology 1988;25:533.

II. LOW TEMPERATURE BIOLOGY ASPECTS

STORAGE MEDIA FOR RED CELLS

O. Åkerblom

During storage in the cold, erythrocytes undergo a number of changes, some of which impair the metabolism and function of the erythrocytes, and set limits to their storage-ability. The aim of erythrocyte preservation is to minimize and delay the deleterious changes.

Fifty years ago British researchers began to study the metabolism and posttransfusion survival of erythrocytes preserved in various citrate-glucose mixtures. In 1943 Loutit and Mollison [1] reported that acidification of the citrate-glucose solution improved the posttransfusion survival of stored red blood cells. The properties of acid-citrate dextrose (ACD) solutions were studied extensively also in the United States, and several reports were published in the "Blood Preservation Issue" of J. Clin. Invest, in July, 1947. These reports confirmed the improved storage-ability of ACD blood: at least 70% of erythrocytes stored for three weeks remain in the circulation of the recipient 24 hours after the transfusion. A three week storage period for ACD blood was thus established, and blood could be stock-piled in blood banks with a reasonably low rate of out-dating. In the same issue of Samuel M. Rapoport published studies on the dimensional, osmotic and chemical changes of erythrocytes stored in citrate-glycose solutions and in ACD [2]. The addition of citric acid to the citrate-glucose preservative solution delayed the fall of glycolytic rate, lactate production, pH, ATP level, and intracellular potassium concentration. Preserved in ACD, the red cells rapidly lost their content of acid soluble organic phosphates (mainly DPG). Rapoport suggested that the maintenance of the functional state of the red cell and of its various characteristics is dependent upon the glycolytic process, and that the ATP is of critical importance for the preservation of this process. He proposed that the viability of the erythrocytes during storage might be related to their content of ATP.

In the fifties, purine nucleosides, e.g. adenosine and inosine, were found to (partly) restore the content of organic phosphates and to improve the posttransfusion red cell survival of stored blood [3]. Also when added from the beginning of storage adenosine retarded the breakdown of red cell organic phosphates and improved the posttransfusion

survival [4]. However, during the break-down of the purine nucleosides, hypoxanthine is formed which upon transfusion is converted into urate. Due to the high amount of purine nucleosides necessary to improve storage-ability significantly, transfusion of a few units of purine nucleoside fortified blood rises the recipient's serum urate level considerably[5].

Nakoa et al reported that although incubation with inosine raised the levels of organic phosphates in outdated red cells, addition of adenine was necessary to elevate the ATP level, and that the rise in ATP was associated with normalization of red cell shape [6] and posttransfusion viability [7]. Simon et al [8] proved that small amounts of adenine, present from the outset of storage, was sufficient to improve the maintenance of ATP and posttransfusion viability. Supplementation of ACD blood with 0.5 mM adenine, extended the storage period to five weeks [8–10]. The amount of adenine was small enough to allow the routine use of ACD-adenine as a blood preservative [11].

Reports in 1967 showed that the 2,3-diphosphoglycerate level correlated negatively with the hemoglobin oxygen affinity [12,13]. This finding favoured the use of the citrate phosphate-dextrose (CPD) solution which is less acid than ACD and therefore gives a better maintenance of DPG and normal hemoglobin oxygen affinity [14]. Another approach was to reevaluate the possible role of inosine in red cell preservation. Incubation with "cock-tails" composed of inosine, pyruvate, inorganic phosphate and adenine was shown to "rejuvenate" stored red cells, and to induce supranormal DPG and ATP levels, and a marked decrease of the hemoglobin affinity for oxygen in fresh and stored cells [15–17]. However, due to practical problems, these findings could not be practized on a large scale. Instead CPD fortified with adenine was introduced in the mid-seventies in order to improve the standard blood preservation [18,19].

Whole blood was hitherto the dominating blood product. However, theoretical considerations as well as practical experience showed that the use of components of blood rather than whole blood would improve transfusion therapy. In addition, the production of packed red cells was large in countries producing plasma for industrial fractionation from whole blood donations. Therefore, methods allowing improved preservation and facilitated transfusion of packed cells was urgently needed. Studies in the mid-forties on resuspension solutions for packed red cells [20] did not lead to practicable methods; multiple blood bag systems were developed much later. The development during the late seventies was much more successful, and in *the eighties*, the use of erythrocytes suspended in additive solutions have in many countries replaced whole blood and packed cell transfusions. These additive solutions contain adenine and glucose in saline with the addition of a non-penetrating agent, e.g. mannitol, to minimize the spontaneous *in vitro* hemolysis during storage. Some of the solutions do also contain other substances, i.e. guanosine, inorganic phosphate, citrate, sorbitol. These solutions

are added to the packed red cells shortly after blood donation and separation of plasma. They improve storageability [21–24] and dilute the red cells sufficiently to allow one unit of red cells to be transfused as rapid as one unit of whole blood [21].

The events taking place in stored red cells may be summarized as follows:

1. Glucose is metabolized to lactate and hydrogen ions, which accumulate in the blood container. The decreasing pH inhibitis successively the phosphofructokinase and hexokinase enzyme reactions, resulting in a decreasing rate of glycolysis during storage.

2. The rephosphorylation of ADP to ATP decreases concomitantly with the decreasing glycolysis rate, resulting in a fall of the ATP concentration and increasing ADP/ATP and AMP/ATP ratios. There is a net loss of adenosine nucleosides due to an irreversible deamination of AMP.

3. The DPG level starts to fall when the pH falls below 7.3 [25]. (Red cells with a pH of 7.3 at 4°C will show pH 6.8 when measured at 37°C [cf 26]). The DPG phosphatase activity is low, therefore the DPG decay is linear until 10% of the original level is reached. The daily loss at 4°C is approximately 0.07 mol/mol hemoglobin [27], corresponding to 8% of the normal level.:

4. At 4°C, the cation pump cannot balance the potassium outflux. As a result, the extracellular potassium concentration increases rapidly during storage, and approaches equilibrium with intracellular potassium after 6 weeks of storage [2]. The loss of erythrocyte potassium is therefore dependent on the extracellular volume, the lowest potassium loss occurring in packed red cells..

5. The posttransfusion erythrocyte survival is dependent on biochemical function and membrane integrity. Subnormal ATP levels correlate with decreased erythrocyte viability [9,11,28]. In stored red cells, the total adenosine nucleotide content rather than ATP is better correlated with viability [29]. The morphology changes occurring are also related to the ATP and posttransfusion survival. Preservatives giving improved ATP maintenance do also delay the equinocyte and spherocyte formation [31]. The decrease of the posttransfusion survival is related to the number of spherocytes and partly the number of equinocytes. Spherocyte formation is associated with loss of membrane surface area [32,33], which will cause irreversible changes, while most equinocytes can be re-transformed to discocytes [29, cf 30].

6. The oxygen affinity of the hemoglobin is closely related to the DPG concentration and increases when the DPG level falls during storage [34,35]. In most transfusion situations this is of no clinical significance, although there may be cases in which a normal (or decreased) oxygen affinity is important. However, preservatives and additive solution are generally designed primarily to prolong ATP maintenance and viability; these factors are usually not allowed to be impaired by efforts to improve the maintenance of DPG and normal oxygen affinity of the hemoglobin.

Most of the changes occurring during storage are reversible upon transfusion. Restoration of ATP, DPG and potassium levels begins immediately after transfusion, and normal concentrations are reached within one or a few days [36]. *In vitro* restoration of ATP, DPG and red cell morphology by incubation with "rejuvenation cock-tails" is accompanied by an improved posttransfusion survival. However, this improvement does not lead to full restoration of red cell viability, indicating that membrane changes, unrelated to the metabolic state of the cell, occur during storage [cf 30].

The effects of the different substances used in the anticoagulant preservatives and in the additive solutions can be summarized as follows:

1. *Trisodium citrate.* The citrate ion does not penetrate the red cell [37], and the plasma concentration in ACD and CPD blood is 20–23 mmol/l. This concentration is much higher than needed for the binding of calcium ions and anticoagulation. Addition of citrate elevates the intracellular pH slightly [38–40]. This has to be considered when relating glycolytic activity and DPG maintenance to the pH of the red cell suspensions and whole blood. Another possible effect of the 20 mM citrate concentration might be to balance the colloidosmotic effect of the red cell hemoglobin, approximately 17 mosm/kg [41], and thus minimize the spontaneous hemolysis of stored red cells.

2. *Citric acid.* Cooling the blood from 37°C to 4°C decreases the dissociation constant of water and thus the hydrogen ion concentration; the pH increases by approximately 0.5 pH unit [cf 26]. The addition of citric acid counteracts the temperature-induced elevation of the pH, and is necessary to avoid the negative effects of an alkaline pH upon the energy status of the erythrocytes: a rapid decrease of the ATP concentration [25], increased AMP/ATP and ADP/ATP ratios, and a marked increase of the breakdown of adenosine nucleotides to hypoxanthine [42].

3. *Glucose* is the main nutritient for the erythrocyte. The amount of glucose present in donor blood is not sufficient for the preservation of red blood cells. CPD-adenine stored red cells consume 5.84± 0.55 mmol glucose per mmol hemoglobin in five weeks [43]. This corresponds to

approximately 0.9–1.2 g of glucose per unit of blood. Standard ACD and CPD solutions provide enough of glucose for several weeks of storage of whole blood. For CPD-adenine packed red cells a five week storage period can be obtained either by leaving 80–90 ml of plasma with the cells, or by adding more glucose to the anticoagulant solution. However, resuspension in an additive solution containing glucose will give a better product and allow storage beyond five weeks.

4. *Adenine.* During storage red blood cells loose adenosine nucleotides due to deamination and degradation of AMP. Adenine is readily taken up by the erythrocyte, and is incorporated into the adenosine nucleotide pool. Adenine (A) reacts with phosphoribosyl diphosphate (PrPP) to form AMP:

$$A + PrPP \rightarrow AMP + PP$$

ATP and ribose 5-phosphate is utilized for the synthesis of PrPP:

$$ATP + rP \rightarrow AMP + PrPP$$

ATP and AMP equilibrate with ADP in the adenylate kinase reaction:

$$ATP + AMP \leftrightarrow 2\ ADP$$

The ADP can then be rephosphorylated to ATP by glycolysis. A maximal effect upon ATP is achieved at an adenine concentration close to 0.5 mM in whole blood, higher concentrations giving less good maintenance of the ATP level [9,27].

An increased ADP/ATP quotient favours the metabolism of 1.3-DPG via the phosphoglycerate kinase reaction and reduces thereby the synthesis of 2.3-DPG. Adenine addition gives a more rapid decline of the DPG level in stored red cells [9]. In CPD blood the DPG decline is closely related to the adenine concentration [27]. In the fresh erythrocyte, the PrPP is present in a very low concentration, but increases rapidly during the first week of storage [44]. This explains why the consumption of adenine is low the first few days of storage, and then more rapid. A standard unit of blood consumes 0.5–0.8 mg of adenine per day, the consumption rate being highest the first two weeks of storage [9,45].

Upon transfusion, the remaining free adenine is metabolized to adenosine nucleotides and nucleic acids in the recipient. A minor part will be excreted unmetabolized. A few per cent of the adenine is metabolized to dioxyadenine (DOA), which may form precipitates in the kidney when excreted. To safely avoid nephrotoxic risks, the dose of adenine given with the transfusion should not exceed 15 mg (0.11 mmol) per kg body weight [46]. This may create a problem in the massive transfusion situation unless the adenine in the preservative or additive solution is kept low. The low content of adenine in the SAGMAN solution, 17 mg, is considered safe for all types of transfusion situations,

although it is suboptimal with regard to the maintenance of ATP [9, 27].

5. *Inorganic phosphate (iP).* The amount of iP in the CPD solution is probably too low to influence the glycolysis of the erythrocyte significantly. Very high levels of iP improves the maintenance of ATP [47]. Production of supernormal DPG cells with nucleosides and pyruvate requires the addition of iP.

6. *Guanosine* is a poorly soluble purine nucleoside, which has been used together with adenine in "ACD-AG" solution [48,49] to give a 0.25–0.50 mM concentration of guanosine in the blood. It is rapidly converted to guanine, which is slowly being converted to xanthine. A small amount of the guanine is converted to guanosine nucleotides. The molar concentration of GTP may rise to 10–15% of the ATP concentration [50]. Guanosine improves the preservation of ATP as well as DPG [50,51], and prolongs the storage period by at least one week.

Transfusion experience and recent toxicological studies in humans have not revealed any untoward effects of guanosine [Förster H, unpublished report 1987]. However, the exact mechanism of action has still to be clarified, as well as the possible risk of supplying the massively transfused patient with the guanine and xanthine formed during storage.

7. *Mannitol* or sorbitol are being used in additive solutions to reduce the spontaneous hemolysis occurring in erythrocytes resuspended in saline solutions. Although a 10 mM concentration of mannitol effectively reduces the *in vitro* hemolysis [52], 29 or 41 mM mannitol or 55 mM sorbitol is currently being used in additive solutions. The mechanism of action is not known in detail. One possible explanation could be that a few red cells become more leaky to cations during storage, and that the colloid-osmotic effect of the hemoglobin, approximately 17 mosm/kg, causes swelling and hemolysis of the cell [41]. Addition of non-penetrating agents such as citrate, mannitol, sorbitol or sucrose in concentrations able to balance the colloid-osmotic effect of the hemoglobin will then minimize the *in vitro* hemolysis during storage.

Several other substances which improve the metabolic state of the red cell have been proposed to be included in the preservative solution, i.e. dihydroxyaceton, bicarbonate, ascorbate, ribose, xylitol, phosphoenolpyruvate. However, due to technical and other problems these substances have found little or no use in the routine blood banking. Also other factors influence the storageability of the red cells i.e. variations of storage temperature, intermittent mixing of the cells during storage, phtalates from the plastic bag, and degree of oxygenation.

The aim of blood preservation in the liquid state is to keep controlled and safe blood components with high degree of efficacy immediately available for transfusion. Erythrocytes resuspended in additive solutions

fulfil this requirement. The extended preservation time, 5–7 weeks, facilitates stockpiling and minimizes the risk of out-dating. However, most red cells are transfused within two or three weeks after the blood donation, and the need for longer storage periods than 5–6 weeks is small. It seems to be more important to minimize the variation in efficacy between the newly prepared red cell unit and the one approaching it's expiration day. Also, to allow the use of the red cell component in chronic anemia as well as for massive transfusion and exchange transfusion, the additive solution should contain minimum amounts of adenine and other additives which may give untoward toxic effects when given in large amounts. Otherwise, the red cells have to be washed before transfusion, which is costly and laborious and reduces the availability.

The CPD SAGMAN system designed by Högman [21,22] was introduced to 100% in our hospital eight years ago. In 1988, SAGMAN erythrocytes were used in 96% of all red cell transfusions in Sweden. It has facilitated the use of blood components according to modern concepts [54–56]. The number of febrile transfusion reactions were reduced by 70% due to the removal of buffy coat. The iso-agglutinin-poor blood group 0 SAGMAN erythrocytes can be transfused in large amounts to patients belonging to other blood groups without risk of serological complications. This facilitates stock-piling since it is no longer quite necessary to keep all blood groups in stock. Finally, large volumes of high-quality plasma are recovered for production of the coagulation Factor VIII and other plasma derivatives.

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PROLONGED STORAGE OF ADULT AND NEWBORN RED CELLS

N. Meyerstein

Introduction

Prolonged storage of red blood cells induces structural and metabolic changes, known as "storage lesion" [1]. This causes impaired cellular function and a decreased survival time after transfusion [2]. Suitable preservation media may maintain ATP contents and improve *in vivo* survival [3]. Recently a new preservation medium was composed by Meryman et al, consisting of a hypotonic solution with respect to non permeant solutes [4]. Ammonium chloride, adenine, glucose, mannitol, citrate and phosphate were included in this medium [4]. Unexpectedly, this medium augmented ATP levels and maintained them above initial values for 9 weeks, as well as prolonged *in vivo* survival at acceptable values, for up to 18 weeks.

In our previous study, we investigated the advantageous effect of the different constituents of this preservation medium as compared with CPDA-1 [5]. It was clearly demonstrated that neither ammonium chloride nor potassium was essential. The important constituent was mannitol, with its known osmotic support [6] and antioxidant activity [6,7] in a hypotonic protein-poor medium. In order to acquire a better understanding of the underlying mechanism, newborn cells, with known impaired antioxidant systems and accelerated *in vivo* senescence were stored in Meryman's medium.

Materials and methods

Blood for each experiment was obtained from 3 healthy adult donors, after informed consent. In addition, cells were obtained from the umbilical cords of 3 mature newborns, immediately after delivery. These samples were collected aseptically in heparinized sterile test tubes. After plasma and leukocytes were discarded, the cells were washed in sterile isotonic saline solution and suspended in Meryman's "solution 6". Final volume was less than 5 ml in each tube. The ratio of storage medium to cells was 1:1. The tubes were stored unstirred for 12 weeks, at 4°C,

opened at weekly intervals for serial determinations and then discarded. All preservation media were prepared and then sterilized by passage through 0.02 μm filters. The osmolality of each solution was determined using a Fiske Osmometer, as follows: Meryman's "solution 6" [4]: 408 mosmols. Its permeant solutes (glucose, adenine and ammonium chloride) contributed only 210 mosmols. The cells were introduced into this solution which gradually became hypotonic as the permeant solutes entered the cells during storage. Thus, the media were hypotonic with respect to non-permeant solutes.

Variables measured

ATP contents were determined as reported previously [8]. 2,3-DPG levels were determined according to Beutler [9]. Both values were expressed as μmoles per gHb. The enzymes for ATP and 2,3-DPG were purchased from Sigma Co., Petah Tikva, Israel. Hemoglobin, hematocrit and osmotic fragility were determined according to Dacie & Lewis [10]. The osmotic fragility was expressed as Median Fragility, i.e. the salt concentration of the hypotonic solution causing 50% hemolysis [10].

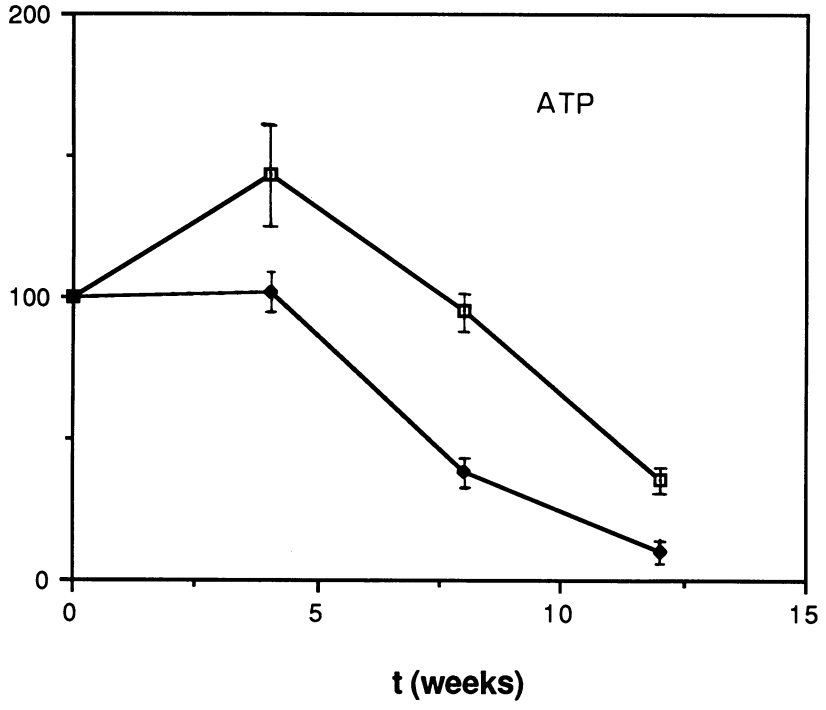

Figure 1. Mean ATP levels ± SEM in units stored in Meryman's "solution 6": ❑ adult cells; ◆ newborn cells. Data represent 3 units in each solution; calculated as percentage of initial values.

Table 1. Effect of prolonged storage on newborn red cells in Meryman's solution.

Storage (weeks)	ATP (% of initial value)	DPG (%)	Free hemoglobin (mg%)	Osmotic fragility (mM)	pH
0	100	100	0	85	6.85
4	102 ± 6.5	9.7 ± 1.7	322 ± 51	114	6.48
8	39 ± 2.7	0	780 ± 117		6.23
12	10 ± 1.6	0	1003 ± 281		

Data are expressed as means ± SEM.

Results

The results of a 12-week storage period in Meryman's "solution 6" are presented in Figure 1 and Table 1. ATP levels were maintained in this medium at initial values for 4 weeks, then declined to 39% at 8 weeks and to 10% at 12 weeks (Fig. 1).

2,3-DPG levels, however, decreased very rapidly to about 10% of their initial value and were subsequently negligible throughout storage (Table 1). Hemolysis, as expressed by free hemoglobin in the preservation media, also increased rapidly, reaching 300 mg% at 4 weeks and then twice this value at 8 weeks. There was also a rapid increase in osmotic fragility, and later on overt hemolysis was noted. Similary, pH decreased rapidly, reaching 6.2 at 8 weeks. When these data are presented parallel to those of adult cells in the same media [5], it is evident that newborn cells are much more sensitive to storage: the initial rise in ATP levels at 4 weeks, observed in adult cells, did not occur in newborn cells at all. The rate of ATP decline, however, was similar in both

Table 2. Effect of prolonged storage on newborn and adult* cells in Meryman's solution.

		Weeks of storage			
		0	4	8	12
ATP	newborn	100%	102 ± 6.5	39 ± 2.7	10 ± 1.6
(%)	adult	100%	143 ± 7.6	95 ± 5.7	36 ± 1.6
DPG	newborn	100%	9.7 ± 1.7	0	0
(%)	adult	100%	35.1 ± 4.9	19 ± 2.6	N.D.
Free hemoglobin	newborn	0	322 ± 51	780 ± 117	1003 ± 281
(mg%)	adult	0	38 ± 15	71 ± 12	377 ± 158

* Data for adult cells presented previously [5].

Data are expressed as means ± SEM.

cell types (Fig. 1). A similar pattern was observed in 2,3-DPG levels and free hemoglobin (Table 2). However, osmotic fragility was similar in both cell types and pH was better maintained in adult cells than in newborn cells.

Discussion

Meryman et al have described a new preservation medium, containing ammonium chloride in hypotonic solutions, supporting high ATP levels and acceptable post-transfusion survival, even at 18 weeks [4]. This medium also contains mannitol, to reduce hemolysis in the absence of plasma proteins, as proven by Högman [11]. Recently, Beutler et al suggested that the mechanism for the beneficial effect of mannitol is more complex than its osmotic support [6] and may involve scavenging of free radicals [7].

Other studies have confirmed the impressive beneficial effect of Meryman's "solution 6" with its amazing increase of up to 140% in initial ATP values during the first 4 weeks in cold storage [5]. These high levels were maintained for at least 8 weeks with low spontaneous hemolysis.

The mechanism responsible for the beneficial effects of Meryman's medium is still unexplained. In order to achieve a better understanding of it, we used newborn cells, known for impaired antioxidant defense systems and accelerated senescence. These cells differ from adult red blood cells in several of their characteristics: most of the hemoglobin is fetal hemoglobin (HbF), a high affinity hemoglobin, and the majority of the enzymatic activities are greater than in adult cells [12]. However, their antioxidant defenses are less efficient, with low activity of glutathione peroxidase and methemoglobin reductase [12]. Newborn cells have a shorter life span than adult cells, a fact which may be correlated with their increased sensitivity to oxidation. As red cell senescence has been likened to *in vitro* cold storage [13], we used newborn cells to evaluate Meryman's medium. We found that after 4 weeks, newborn cells still had high ATP levels, close to initial values. However, this is lower than in adult cells, in which ATP levels increase to about 140%, as is shown in Meryman's data. The rate of ATP decline is the same in both cell types. It should be mentioned here that ATP levels in newborn cells are similar to those in adult cells, some times even higher [12,14], but are considered to be more labile during incubation. Reduced glutathione (GSH) levels are higher in newborn than in adult cells, but again are defined as more "labile" [12]. This lability may be interpreted as being related to impaired efficiency of antioxidant defenses in the newborn [12]. This phenomenon is also comparable to *in vitro* aging: Lachant et al [15] have shown that in spite of normal GSH levels, stored cells are more sensitive to oxidative stress. This sensitivity is demonstrated by increased oxidation of hemoglobin (demonstrated by Heinz Bodies) and

by decreased GSH [15]. In this study, impaired red cell survival was significantly related to the GSH stability 24 hours after transfusion.

As has been shown in previous studies, newborn cells were found to be inferior to adult cells in storage in CPDA medium [14]: these cells had lower ATP and DPG levels, a fact which usually indicates poor (although sometimes only transient) post-transfusion function. The newborn cells manifest increased osmotic fragility and a greater hemoglobin release in the media, suggesting membrane damage.

Storage of newborn red cells also has practical implications: these cells have been considered in the past for autologous transfusion, in ACD [16] and CPD [17]. Previous studies in CPDA-1 have shown that newborn cells which may be obtained from placentas and stored for several weeks are more affected by storage than adult cells [14]. The "storage lesion" in these cells was partly reversible, as in adult cells, by incubation with adenosine, i.e. the cells could be rejuvenated by this incubation.

It is thus tempting to speculate that antioxidant protection is deficient in these cells *in vivo* as well as *in vitro*. This finding may reinforce the possible role of antioxidant activity in Meryman's medium.

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EXTENDED STORAGE OF (WASHED) RED CELLS AT 4°C

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There are three general procedures for the storage of human red cells for transfusion:

1. refrigerated storage in the original anticoagulant solution;
2. refrigerated storage after separation of the red cells from the anticoagulant solution and resuspension of the cells in a solution specifically designed for red cell storage;
3. freezing of red cells in glycerol solution.

In the United States the standard anticoagulation solution is CPDA-1. Red cells collected are stored in CPDA-1 solution (see Table 1) have a shelf life of 35 days. Although a number of investigators over the last two decades have reported possible benefits from anticoagulant solutions at higher pH, [1–3] solutions in actual use are all at low pH because of manufacturing considerations. When a solution containing both electrolyte and glucose is autoclaved at pH above 6.8, the glucose caramelizes.

An alternate procedure is to draw the whole blood into CPDA-1, remove most of the plasma and platelets, then resuspend the red cells in a solution specifically designed for red cell storage. Two solutions, ADSOL and Nutricell, (Table 1) have been licensed for this purpose. These solutions are also at low pH. Red cells resuspended in these solutions have a shelf life of 42 days.

In red cells suspended in any of the three solutions described above, the concentration of adenosine triphosphate (ATP), which bears a loose relationship to membrane fluidity, red cell shape change and *in vivo* survival, rises briefly then falls progressively during storage, reaching between 40 and 60% of initial value at the end of the storage period. 2,3-diphosphoglycerate (2,3-DPG), which is associated with the ability of the hemoglobin to deliver oxygen to the tissues, falls rapidly after 3 or 4 days of storage and by 10 days is approaching zero.

The proportion of cells that will continue to circulate after transfusion also falls during storage. Shelf life has been determined on the basis of this, the general rule being that 75% of the cells originally collected should still be circulating 24 hours after transfusion. The cells that do

Table 1. Composition of red cell suspending solutions, mM.

	CPDA-1	ADSOL	Nutricell	ARC6	ARC9C	ARC8
NaCl		154.0	70.1			
Adenine	2.0	2.0	2.2	2.0	2.0	2.0
Glucose	161.0	111.0	55.0	110.0	177.0	138.0
Mannitol		41.2		55.0		
MaCitrate	89.6		20.0	17.9	27.2	33.3
Citric acid	15.6		2.0			
NaH_2PO_4	16.1		20.0	14.7		3.26
Na_2HPO_4				25.8	20.0	11.6
NH_4Cl				50.0		
pH	5.7	5.5	5.8	7.1	7.5	7.4
Osmolality	323	342	244	199	121	126

This table summarizes the composition of solutions referred to in the text. Osmolality is presented in milliosmoles and refers only to the non-penetrating consitutents, glucose being assumed to penetrate the red cell [12].

not circulate are presumed to be those that have lost membrane fluidity and undergone shape changes that render them unable to pass through narrow channels in the spleen and liver. Both ATP concentration and the proportion of cells with abnormal morphology therefore provide some indication of probable 24 hour *in vivo* survival [4].

Some of the shape changes that cells undergo during storage are reversible. Some of these reversible cells may revert to normal morphology after transfusion before they pass through the filters of the spleen and liver. Others may be filtered out before they have had an opportunity to recover. It is possible to rejuvenate stored cells so that, in theory at least, all potentially reversible shape changes are reversed prior to transfusion and the *in vivo* survival is improved [4]. Another virtue of *in vitro* rejuvenation, in fact the basis for its original use, is the regeneration of 2,3-DPG levels so that the cells will deliver oxygen immediately following transfusion [5].

Since current rejuvenating solutions are not suitable for transfusion, rejuvenated cells must be washed prior to transfusion. Since the closed system will have been entered to introduce and remove the rejuvenating solution, federal law requires that the cells be transfused within 24 hours because of the risk of bacterial contamination. If the closed system were not entered during rejuvenation and washing, the 24-hour limit would not be applied provided a satisfactory resuspension solution were also available. New devices recently licensed make it possible to join two pieces of transfer tubing without risk of contamination so that the contamination obstacle can now be surmounted. However, the saline glucose

solutions normally used for washing are not suitable for storage beyond 24 hours because of rapidly increasing hemolysis. No transfusable solution capable of providing prolonged storage following washing has been reported. For this reason, rejuvenation is always followed by freezing.

For freezing, red cells are suspended in a lactate solution containing glycerol which protects them from freezing injury [6]. Cells can then be stored at –80°C for many years. Following thawing, the glycerol is washed from the cells leaving the cells suspended in a glucose saline solution. The same restrictions previously described now apply and the cells must be transfused within 24 hours. The advantage of freezing following rejuvenation is merely that thawing and deglycerolization can be done electively rather than being mandatory within 24 hours. We have investigated the possibility of extending 4°C storage of red cells beyond the 42 days achieved by ADSOL and Nutricell and have also attempted to design a solution suitable for the storage of washed cells.

Our first indication that refrigerated storage might be substantially extended resulted from experiments stimulated by reports that part of the progressive storage lesion consisted in the development of echinocytes leading ultimately to the budding off of vesicles from the tips of the spicules [7]. We reasoned that this loss of membrane material would decrease the surface-to-volume ratio and increase the rigidity of the cells, thereby increasing the probability that they would be filtered out of the circulation following transfusion and contribute to poor *in vivo* survival. We further reasoned that storing the cells in a hypotonic medium would increase their surface tension and tend to oppose the formation of echinocytes. To test this hypothesis experimentally, red cells were stored in a solution of approximately 150 milliosmoles to maximize cell swelling.

Early studies by Davson [8] showed that red cell membrane permeability is increased as the ionic concentration falls below 100 mM of electrolyte. We had subsequently shown that one could achieve the osmotic effects of hypotonicity, i.e., cell swelling, without a corresponding reduction in ionic concentration by substituting ammonium chloride or ammonium acetate for non-diffuseable electrolytes [9]. The ammonium ion, NH_3^+, in aqueous solution is always in equilibrium with a small amount of NH_4. This neutral molecule passes readily across the membrane where it will lose a proton to re-establish normal dissociation. Thus, an ammonium salt that includes a penetrating anion will freely penetrate the red cell membrane and, as a result, exert no net osmotic effect.

In order to store red cells in a hypotonic medium without the risk of alterations in membrane permeability we therefore added 100 mM ammonium acetate or ammonium chloride to the otherwise hypotonic suspending solution (ARC6, Table 1). To our surprise, although 2,3-DPG, as expected, fell rapidly, ATP rose steadily during subsequent storage, reaching 150% or more of original value in roughly four weeks, following which there was a progressive decline reaching the initial level in roughly ten weeks. Subsequent *in vivo* studies showed a 24-hour *in vivo*

survival in excess of 75% after 100 days (14 weeks) of storage. Both the hypotonicity and the ammonium chloride were shown to be beneficial to ATP maintenance when employed alone and with cumulative benefit when combined [10].

At the time these observations were made, the results were unexpected and the mechanism a mystery. Subsequent review of the pathways of carbohydrate metabolism, particularly the elegant studies of Rapaport [11], revealed that ammonium is an activator of the enzyme phosphofructokinase (PFK) which converts fructose-6-phosphate to fructose 1, 6 diphosphate at pH above 7. Most of the enzymes of carbohydrate metabolism are "pass-through" enzymes that process substrate whenever it is presented to them. PFK is an allosteric enzyme that can be activated or inhibited by compounds other than its substrates. Furthermore, it is positioned early in the glycolytic pathway, thus functioning as a gatekeeper for glucose metabolism and the subsequent synthesis of ATP. It is not unreasonable to propose therefore that the activation of PFK by ammonium to an extent far in excess of that possible *in vivo* is responsible for the increase of ATP to a supra-normal level. If this were the explanation for the effectiveness of the ammonium chloride, this would imply that the maintenance of ATP during storage could be substantially improved by any factor that activates PFK. Since the inclusion of ammonium salts in a red cell suspension designed for transfusion is of questionable acceptability, we directed our attention at other ways by which phosphofructokinase could be activated.

Other activators of PFK include AMP, sulfate, potassium, inorganic phosphate (Pi) and magnesium [11]. With the exception of Pi, none of these activators were found to be effective in stimulating ATP synthesis in intact red cells during 4°C storage, possibly because of the impermeability of the red cell membrane. Pi, on the other hand, was very effective in stimulating ATP synthesis at a rate and to a level proportional to the Pi concentration up to a saturation level at about 40 mM.

Inhibitors of PFK include ATP itself, citrate and 2,3-DPG. Of these, only citrate was amenable to manipulate but its presence or absence in the incubation solution was without effect on ATP or 2,3-DPG maintenance possibly, in this case as well, because of its failure to penetrate the red cell membrane. Our attention was therefore focused entirely on manipulation of the phosphate concentration and its presentation to the red cells.

Phosphate, in a red cell storage solution, could perform at least three essential functions. First, it can activate PFK; second, it is itself one of the substrates for the synthesis of ATP and 2,3-DPG; third, it is an effective buffer in the range pH 7 to 8 necessary for efficient glycolysis. In designing a practical, transfuseable, red cell storage solution, we felt restricted in the extent to which we might elevate the phosphate concentration above that in current solutions because of concern on the part of clinicians, especially when transfusing patients with renal problems. We

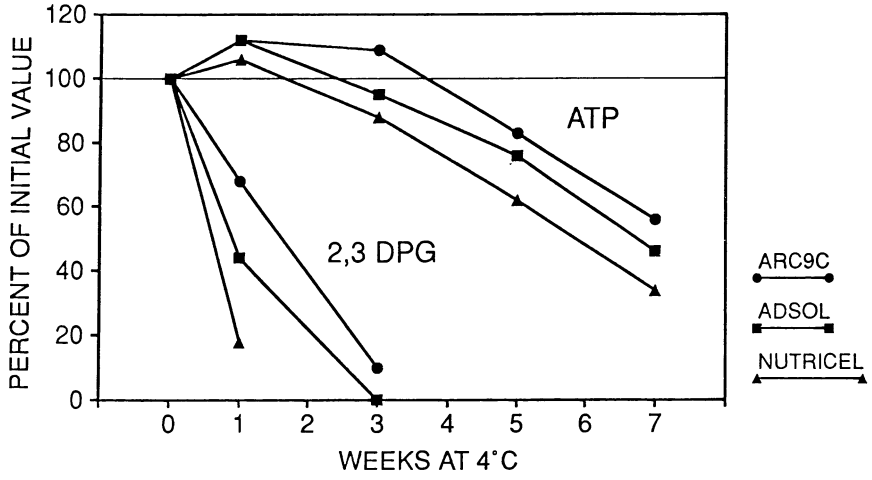

Figure 1. Red cells were collected in CPDA-1, platelet-rich plasma removed and the cells were resuspendid in 100 ml of either Nutricell, ADSOL or ARC9C.

therefore designed our solution so that the quantity of phosphate in the final cell suspension was comparable to that in a unit resuspended in Nutricell which has the highest phosphate concentration of currently licensed solutions.

Once NH_4 was eliminated, our solution (ARC9C, Table 1) had then essentially the same constituents as CPDA-1, the major difference being the elevated pH and the hypotonicity of non-penetrating constituents. When used for the resuspension of red cells collected in CPDA-1, the elevation of ATP was, not surprisingly, moderately prolonged in comparison to cells resuspended in ADSOL or Nutricell (Figure 1).

Coincidental with these studies, we were aware of increasing concern on the part of blood bankers regarding the contamination of red cells by transmissible viruses, especially the hepatitis B virus (HBV) and the human immunotropic virus (HIV). Proposed approaches to viral inactivation or depletion will inevitably require washing of the red cells either for physical depletion of virus particles or to remove inactivating agents and their byproducts. This expectation, plus the existing need for a resuspension solution for rejuvenated or deglycerolized red cells, led us to explore the application of our storage solution to the resuspension of washed cells.

Our initial studies of the influence of phosphate at pH above 7.0 on ATP synthesis during storage had revealed that ATP elevation and maintenance during storage was not only proportional to phosphate concentration but also inversely proportional to hematocrit, raising the question whether the relationship was really with concentration or rather with the absolute quantity of phosphate present. Experiments designed to test this, although not unequivocal, suggested that the latter

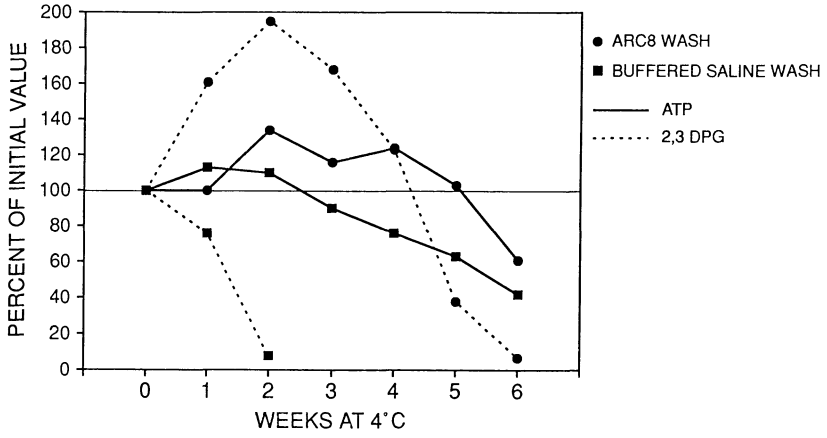

Figure 2. Red cells were collected in CPDA-1, platelet-rich plasma removed and the remaining cell suspension divided into two equal aliquots. One was washed in phosphate-buffered isotonic saline, the other in ARC8. Both were stored at 4°C in ARC8.

might be the case. This in turn, implied that, whether or not activation of PFK was indeed a factor, this requirement could be satisfied by modest amounts of phosphate and further increases provided benefit through increased buffering capacity and, as a result, prolonged maintenance of pH during storage.

When a high pH solution is added to red cells in CPDA-1, the pH generally falls because of the buffering capacity of hemoglobin. To maintain the pH can require an unacceptable high concentration of buffer. However, if the cells are repeatedly washed with a pH 7.4 solution, during the course of the wash the hemoglobin should be raised to that pH and should reach equilibrium with the external buffer. This means that, at the end of the wash, both the hemoglobin and the extracellular buffer will participate in the maintenance of pH during subsequent storage and postpone the fall in pH that results from the accumulation of lactic acid during red cell metabolism which, in turn, inhibits glycolysis leading to a fall in ATP and 2,3-DPG.

Cells washed and stored in ARC8 (Table 1) at pH 7.4 did achieve significantly higher ATP levels during storage than when washed in isotonic saline buffered to pH 7.4 with phosphate and resuspended in ARC8, with the ATP on average remaining above initial value until 5 weeks post wash (Figure 2). More surprising, however, was the rise and continued maintenance of 2,3-DPG, which we attribute to the elevation of pH achieved by repeated washing with a buffered solution at pH 7.4.

We are therefore reporting a washing and resuspension protocol in which red cells are washed with a phosphate or phosphate-citrate solution at pH 7.4 and resuspended in a solution at comparable pH containing phosphate, citrate, glucose and adenine with a total osmolality of non-penetrating solutes of approximately 125 mOsm. Such a

protocol yields red cells in which both ATP and 2,3-DPG are maintained at levels above normal for approximately four to six weeks at 4°C. *In vivo* survival assays are in progress.

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STORAGE OF PLATELET CONCENTRATES IN PLASMA-FREE SYNTHETIC MEDIA

S. Holme

Background

Over the last ten years in the United States, the rate of increased use of platelets and plasma has greatly exceeded that of red cells. To meet this demand, a major objective of the American Red Cross Blood Services has been to achieve maximal yield of these components with minimal additional cost. Since platelets can only be stored for 5 days with 50-60 mL of plasma, issues of prolongation of shelf life while maintaining product quality is an important goal. The use of a plasma-free additive solution for storage of platelets offers the opportunity to achieve these objectives. Specifically, there are several potential advantages to storing platelets in a plasma-free synthetic medium:

1. about 50 mL of plasma per unit collected may be saved,
2. transfusion reactions caused by non-compatible plasma proteins may be avoided, and
3. quality may be improved, which will decrease the number of units transfused.

Historical review

The idea of storing platelets in a synthetic medium is not new. In the 1950s, Tullis [1] described storage of platelets in a salt solution fortified with sodium acetate and gelatin. Baldini [2] published in 1960 survival studies of platelets suspended and stored in a phosphate-buffered salt solution containing glucose and plasma. However, the survival of these platelets was short, less than 20 hours. A contributable cause for the poor viability was that storage was carried out at 4°C rather thn at room temperature. Storage of *plasma*-suspended platelets at 4°C has been shown to result in irreversible disc-to-sphere transformation with poor post-transfusion survival [3].

Recently, there has been a renewal of interest in storage of platelets in an artificial plasma-free medium. Not only is this partly due to the need to maximize plasma recovery, but, more recently, it has become

Table 1. Platelet storage media (components in mM)

	Histidine Tyrode (Rock [3])	**Phosphate Tyrode (Rock [3])**	**PAS (Holme [7])**	**PSM 1-pH (Murphy [16])**	**Plasma-lyte-A (Rock [17])**
NaCl	103.0	103.0	110.0	98.0	90.0
KCl	2.4	2.4	5.1	5.0	5.0
$CaCl_2.2H_2O$	1.8	1.8	1.7	–	–
$MgCl_2.6H_2O$	0.9	0.9	–	–	–
$MgSO_4$	–	–	0.8	–	–
Na_3.citrate	11.0	11.0	15.2	23.0	–
Citric acid	1.9	1.9	2.7	–	–
$NaHCO_3$	23.2	10.4	35.0	–	–
$NaH_2PO_4H_2O$	–	23.9	2.7	25.0	–
Histidine	21.9	–	–	–	–
Na.acetate	–	–	–	–	27.0
Na.gluconate	–	–	–	–	23.0
Glucose	22.0	22.0	35.5	–	–

clear that it is also possible to improve the quality of the platelet product as well. In 1985, Rock and Adams [4] published a study in Transfusion which suggested that platelets could be stored succesfully for 3 days in a modified Tyrode solution with citrate and glucose and with buffer(s) (histidine, phosphate, bicarbonate, Table 1). Storage was carried out in first-generation platelet containers (PL-146) at room temperature. The authors showed that platelets stored in these nearly plasma-free suspending media (approximately 10 mL of plasma was left after extraction) maintained good respiratory activity and aggregation response, similar to what was observed with storage in CPD-plasma. However, a major problem with storage of platelets in plasma-free media surfaced with these studies. With storage of platelets in the modified Tyrode solution *without* addition of histidine or phosphate buffer, a marked drop in pH was observed during storage, reaching 6.1 after 3 days. Addition of histidine and bicarbonate or phosphate prevented some of this pH fall, with levels of 6.7 and 6.4 after 3 days of storage, respectively. Since fall in pH to levels below 6.7 with storage of platelets has been associated with marked morphological alterations [5] with complete loss of viability at pH below 6.1 [3], this represented a serious problem for storage beyond 3 days in these solutions.

Studies by Murphy and coworkers have shown that the cause of the pH fall with room temperature storage of platelet concentrates is the continuous, steady production of lactic acid by metabolism of glucose [3]. Surprisingly, this occurs despite an adequate supply of oxygen since it also takes place during storage in second-generation, highly oxygen-

permeable containers [6]. With platelets suspended in CPD-plasma, fall in pH occurs when lactate levels exceed 20-25 mM when all the bicarbonate buffering capacity of plasma has been exhausted. In order to avoid this problem, two approaches have been taken in the development of synthetic media:

1. use of glucose, but with increased medium buffering capacity to neutralize the lactic acid,
2. storage in a glucose-free medium to avoid lactic acid production.

Development of a synthetic medium with glucose and with sodium bicarbonate as buffer

Based on a systematic investigation over the last three years, our laboratory has arrived at a composition of a synthetic medium (PAS) which is listed in Table 1. It differs from Rock's modified Tyrode solution in that the amount of KCl has been doubled and that more glucose, sodium bicarbonate, and citrate has been added. In our hands, 5 mM of KCl was the optimal concentration for an ionically balanced medium as defined by a better preservation of platelet volume and response to hypotonic shock during storage. The increased amounts of glucose, sodium bicarbonate, and citrate were necessary to sustain normal energy metabolism and to prevent pH fall and clumping with storage beyond 3 days. The addition of glucose to this medium was essential to allow maintenance of normal ATP levels and platelet function beyond storage of 5 days [7]. Sodium bicarbonate rather than phosphate was used as a buffering system since it was observed that the latter markedly stimulated glycolysis with increased lactic acid output causing a fall in pH. The effect of phosphate on glycolysis has previously been reported for red cells [8].

Extensive *in vitro* and *in vivo* studies with platelets stored in PAS has been carried out. In summary, these studies have shown that various platelet *in vitro* properties are better maintained in PAS as compared in CPDA-1 plasma, even when sodium bicarbonate was added to the latter in order to prevent pH fall with prolonged storage [7]. The various *in vitro* platelet properties such as ATP content, hypotonic shock response (HSR), extent of shape change with ADP at day 10 of storage in PAS were similar to or better than respective results for CPD-plasma at day 7 of storage. However, it was also clear from this study that a substantial deterioration of platelet quality took place in both media with increasing storage duration.

In vivo studies have been performed which confirmed these *in vitro* results [9]. In a recent study [10] a total of 25 paired studies comparing PAS with CPDA-1 plasma with 5 days (n = 5), 7 days (n = 10), 10 days (n = 5), and 14 days (n = 5) of storage were carried out. In addition, 10 control studies using freshly prepared CPD-plasma PC were performed. Superior percentage recoveries and longer survivals were found for platelets stored in the synthetic medium when compared to CPDA-1

plasma. A parallel reduction was also noted in both survival and recovery with increasing storage duration. Since platelet availability to the recipient may be defined in terms of both recovery and survival, it was logical to quantify the platelet availability as the area under the platelet survival curve (post-transfusion efficacy). Analogous with previous *in vitro* results, it was found that platelets stored for 10 days in PAS had post-transfusion efficacy similar to platelets stored for 7 days in CPDA-1 plasma. Again, it was clear that substantial loss of *in vivo* viability took place during storage: in terms of post-transfusion efficacy, platelets stored for 7 days in PAS and for 5 days in CPDA-1 plasma were only 70% and 63% as effective as freshly prepared PC.

Significant correlations between *in vitro* parameters (ATP and lactate levels, HSR and discoid shape) and post-transfusion efficacy were found in these studies. Interestingly, the best *in vitro* predictor of *in vivo* viability was the rate of lactate production, r = –0.79. This was observed independent of the storage period. This inverse relationship between *in vivo* viability and lactate production, has been observed in several studies [11,12] and suggests that an elevated platelet metabolic activity may be an important factor contributing to the loss of viability during storage.

Bode and Miller have suggested that platelet storage lesion is caused by activation or stimulation [13,14]. We have speculated that platelet activation may increase the level of metabolic activity and, thereby, "exhaust" the performance of the respiratory system in providing ATP to sustain various cellular functions related to maintenance of viability and function [10]. In order to test this, our laboratory in collaboration with Bode, has stored platelets under conditions where the potential for activation has been reduced. PC were stored in PAS to which inhibitors of platelet activation, PGE_1 (300 μM) + theophylline (1.9 mM), had been added. Previously, Bode and Miller have shown that platelets stored in CPD-plasma in the presence of PGE_1 + theophylline have improved maintenance of *in vitro* characteristics with prolonged storage [13]. In addition, storage was carried out in PL-732 containers in which the surface-to-volume ratio had been reduced by 40%. This was done to reduce the likelihood of platelet activation by collisions with the container walls. It was observed that the metabolic rate was markedly reduced under these storage conditions as reflected by a decrease of 16% in oxygen consumption and a decrease of 31% of lactate production on day 1 of storage [15]. Furthermore, the reduced metabolic rate was related to improved maintenance of respiratory activity, ATP levels, GP1b levels, and other *in vitro* properties believed to reflect platelet viability during prolonged storage. These results suggest that an improvement in PC quality and prolongation of shelf life may be achieved by use of inhibitors of platelet activation, thereby reducing the metabolic activity of the platelets during storage.

A medium such as PAS, containing glucose and bicarbonate, may pose some manufacturing problems. At a pH 7.2-7.4, glucose caramelizes with

heat sterilization, and bicarbonate in solution at this pH is not stable. However, these problems may be solved by storage of the sodium bicarbonate solution at alkaline pH (where it is stable) separate from the remaining solution and combining these two solutions at the time when the platelet concentrate is prepared.

Storage of platelets in glucose-free additive solutions

Due to the concerns described above, the possibility of storage in a glucose-free medium has been explored by several workers [16,17]. Based on previous work conducted by Murphy and co-workers [6], it has been suggested that platelets derive 85% of their energy (ATP regeneration) by respiration. The ratio of the amount of glucose consumed to the amount of lactate produced also suggested that almost all the glucose utilized was converted by glycolysis to lactic acid.

This indicated that substrates other than glucose, perhaps fatty or amino acids, provided most of the energy for the platelets during storage. Recent studies by Murphy and co-workers have suggested that major oxidative substrates may be fatty acids [18]. Our laboratory has investigated the possibility of storing platelets in a glucose-free medium to which amino or fatty acids have been added [19]. However, despite numerous attempts using various combinations of fatty (linoleic, oleic) and amino acids (glutamine), little or no improvement in platelet quality (ATP levels, morphology, hypotonic shock, respiratory activity and response to ADP stimulation) has been obtained as compared to storage in a control medium containing no substrates for energy metabolism. On the other hand, the addition of glucose to the control medium has been found to markedly improve the platelet quality as observed at 5 and 10 days of storage [7,19]. Interestingly, platelets stored in the substrate-free control medium had not completely lost their viability as suggested by the *in vivo* parameters after 5 days of storage, which suggests that platelets may be able to obtain energy through respiration using an internal substrate.

Other workers have also stored platelets in artificial media without dextrose. However, some dextrose, approximately 3.2 mM, is present in these "glucose-free" media due to plasma carryover from the preparation of the concentrates. Adams and Rock have reported that platelets stored for 5 days in Plasmalyte-A (see Table 1) with citrate, but no glucose added, had similar functional characteristics as platelets stored in the same medium to which glucose has been added [17]. Apparently, the amount of glucose was sufficient to maintain normal energy metabolism for 5 days of storage as the amounts of glucose consumed and lactate produced by the platelets were the same in Plasmalyte-A with and without glucose. Interestingly, there was a substantial decrease in the amount of lactate produced by the platelets in Plasmalyte-A as compared to CPD-plasma. This explains why no fall in pH was observed with 5 days of stor-

age in Plasmalyte-A since this medium does not have significant buffering capacity.

We have confirmed Rock and Adams findings in our laboratory. Table 2 shows the results of a paired study in which Plasmalyte-A (with glucose and citrate) was compared to our synthetic medium (PAS) using CPD-plasma as control. The final synthetic resuspending media contained 5-10 ml carryover plasma. The amount of lactate in Plasmalyte-A after 5 days of PC storage was only 5.4 mM compared to 8.3 mM in PAS. Both levels are substantially less than that found for PC in CPD-plasma, 12.2 mM. Since the decreased lactate production was accompanied by a significantly increased rate of platelet oxygen consumption in Plasmalyte-A, it is suggested that more energy production was achieved by respiration and less by glycolysis by the platelets suspended in this medium. This observation may be of major importance in the development of synthetic storage media for platelets since it points out the possibility of lessening the amount of glucose consumed and lactate produced by the platelets during storage by using appropriate modifications of the medium in order to convert energy metabolism from glycolysis to respiration. A substantial decrease in the glycolytic rate may eliminate the need for glucose and a buffering system which, as pointed out earlier, is a combination that poses some problems in the manufacture of storage solutions.

Murphy has developed a glucose-free, phosphate-containing medium (PSM) which allows for steam sterilization at neutral pH and prevents excessive pH fall with storage up to 10 days [16]. As shown in Table 1, this medium contains a substantial amount of phosphate (25 mM), which is used to buffer lactic acid produced from metabolism of glucose present in "carryover" plasma. Preliminary unpaired studies suggested

Table 2. Comparison of PC stored in Plasmalyte-A (with citrate and glucose) with PC stored in PAS.

	Plasmalyte-A with citrate and glucose	**PAS**	**CPD-plasma**
pH	6.9 ± 0.1[1]	7.2 ± 0.2	7.2 ± 0.1
$C(O_2)$nmoles/min/10^{11} plts	1.6 ± 0.4[1]	1.2 ± 0.3	1.2 ± 0.3
Lactate, mM	5.4 ± 1.1[1]	8.3 ± 1.6	12.1 ± 3.1
HSR[2]	68 ± 17	71 ± 14	76 ± 13
ESC[3]	15 ± 5	16 ± 4	12 ± 5

1. Significant difference (paired t-test, $p < 0.05$).
2. Hypotonic shock response (measured photometrically).
3. Extend of shape change with ADP (measured photometrically as % increase in O.D.).

that storage of platelets in PSM gave similar *in vivo* viability results as with storage in plasma [16]. However, a recent confirmatory study involving three laboratories, and in which paired studies were conducted with platelets from each donor stored in both PSM and plasma, showed poorer *in vivo* results with PSM-suspended platelets with 5 days of storage [20]. The reduced viability of platelets in PSM was related to a 50% reduction in the response to hypotonic shock and a 9% increase in mean cell volume during storage which may suggest a loss in the integrity of the platelet membrane or to ATP-dependent Na/K pump activity. The latter may be a likely explanation since it was demonstrated by our laboratory that the loss of platelet viability (post-transfusion efficacy) in PSM was related to a failure in maintenance of normal adenine nucleotide levels ($r = -0.86$). This may indicate that the amount of glucose present in carryover plasma was not sufficient to sustain the metabolic rate of the platelets, which appears to be substantially elevated by the phosphate concentration present in this medium.

After only 1 day of storage, most of the glucose from carryover plasma (approximately 3.2 mM) was consumed and converted to lactic acid (6.3 mM) with platelets suspended in PSM. This glycolytic rate is two to three times higher than found with platelets stored in Plasmalyte-A or in PAS, which show lactate levels of 1.8 and 2.8 mM respectively, after 1 day of storage. Since the probable cause for this increased rate of glucose consumption in PSM is phosphate, which is known to stimulate glycolysis, it appears that its use as buffer may not be appropriate in a platelet preservation medium.

Possible directions for futher development of platelet preservation media

Although it may be unlikely that PGE_1 and theophylline will be used in practical, useful, artificial platelet media, their effect demonstrates the potential of further improvement in platelet quality. The basis for this appears to be reduction in metabolic activity by using storage conditions where platelet activation/stimulation is minimized. Another possibility, as shown by the recent promising storage studies using Plasmalyte-A [17,21], is to modify the components of the medium in order to reduce the glycolytic rate and, thus, the output of lactic acid by glycolysis, thereby abolishing the need for glucose and a buffering system.

Some potential problems may surface with the introduction of platelet synthetic storage media. Currently, special additive solutions are used for the storage of red cells. The introduction of a special *platelet* additive solution will mean that a new pack containing this solution has to be manufactured which could mean extra cost and labor for the blood bank industry. In order to avoid this, we have modified our platelet additive solution so that it may be used also for storage of red cells. Preliminary *in vitro* and *in vivo* studies using this combined storage solution have

indicated good maintenance of both platelet and red cell quality similar to storage in CPD-plasma (platelets) and in ADSOL (red cells) [22].

Acknowledgements

The authors thank Dr. A. Heaton for helpful suggestions in the preparation of this manuscript, and Diane Garvin for typing it.

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SEPARATION AND PURIFICATION OF COLD INSOLUBLE GLOBULINES

C.Th. Smit Sibinga, P.C. Das

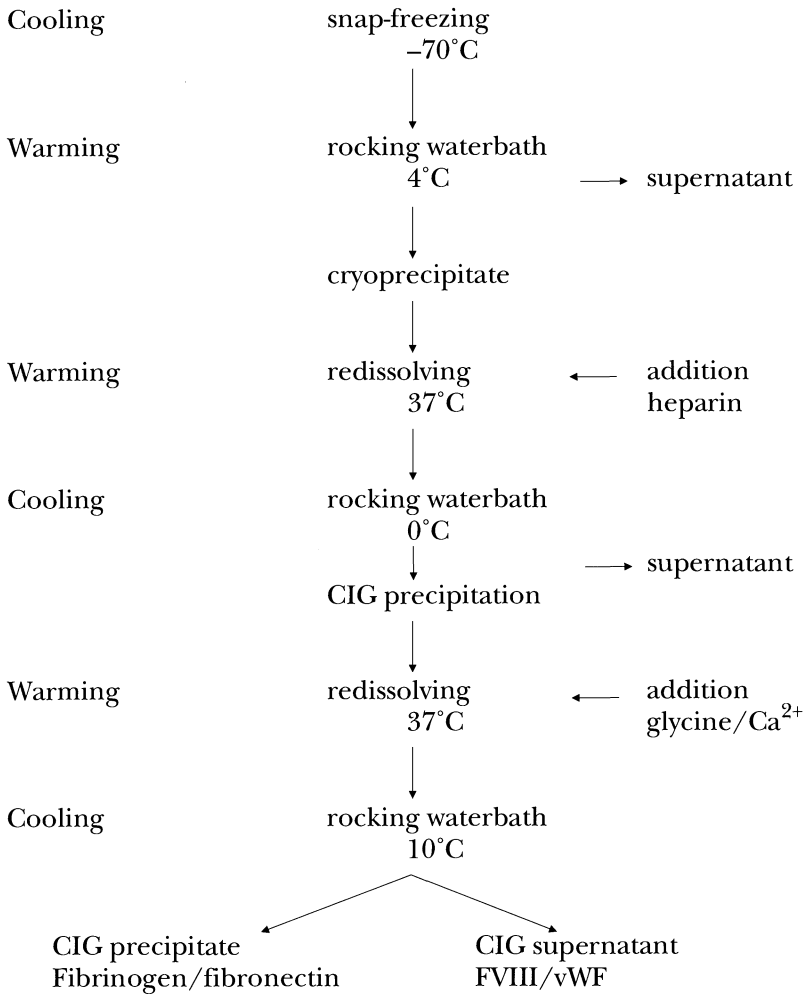
The cold insoluble globulins (CIG) fraction of human plasma proteins comprises a significant amount of the total protein mass and consists of the bulk proteins fibrinogen and fibronectin, and the trace proteins factor VIII/von Willebrand factor complex and factor XIII (fibrin stabilizing factor).

Each of these globulins has the characteristic of changing face over a specific temperature range, thus allowing separation from the remaining plasma proteins. There are certain substances that enhance phase change or precipitability of these globulins, for instance heparin and glycine. The temperature ranges over which the different globulins of the CIG fraction change phase show a considerable overlap, although each seems to have an optimum for gel formation. Separation and purification of the CIG can be achieved basically through simple physical principles of cooling and warming, process which is usually called cryoprecipitation. The transition of the sol phase to the gel phase and back takes place at low temperatures, close to 0°C. At that point plasma supercooling and ice crystal formation do also occur and may adversely affect the preservation of molecular integrity and biological fraction of the respective CIG's. Therefore, both the cooling (freezing) and the warming (thawing) process need to be carefully conditioned and controlled in order to recover optimal qualities and quantities of CIG's.

Conditions of cooling

When cooling plasma to separate and purify CIG's, two major areas of critical attention are to be distinguished: The medium and cooling process persé (Table 1).

The medium – Plasma is about 90% water and around 7% proteins. To recover plasma anticoagulation is needed, which is traditionally achieved by using citrate to chelate the divalent cation Ca^{2+}. Most anticoagulant solutions also contain preservatives such as phosphate, dextrose and adenine, which affect the ionic strength of the medium. The citrate concentration is usually enforced by the addition of citric



Scheme 1. Separation and purification of CIG from plasma.

Table 1. Conditions of cooling (freezing).

Medium	pH calcium concentration ionic strength
Cooling	rate heat exchange eutectic point temperature control time

Table 2. Stepwise recovery of factor VIII (IU/kg) from ACD and CPD plasma. The recovery of each step from CPD plasma differs significantly (s) from ACD plasma.

	Factor VIII, IU/kg		
	ACD	**CPD**	
Oxford 5 l packs			
Plasma cores	710	810	s
Cryo extract	325	367	s
Final product	210	256	s

From: Smith JK, Evans DR. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

acid, determining the pH of the medium. Smith and Evans [1] illustrated the effect of both ACD and CPD on the recovery of the trace CIG factor VIII, by analyzing the separation and purification procedure stepwise (Table 2). The less acid CPD containing medium showed a significantly greater recovery of functional FVIII:C as compared to ACD over the respective steps of plasma freezing (core samples), cryoprecipitate extraction and final purified product. Prowse [2] demonstrated the favourable effect of restoring the divalent cation concentration (Ca^{2+}) to near normal or reversely lowering the citrate concentration in plasma, on the recovery of the trace CIG FVIII:C. This was compared to the recovery from plasma anticoagulated with heparin to allow the medium to keep a normal Ca^{2+} concentration (Table 3 and 4). Addition of 10mM CaCl2 to CPD plasma showed a significant restorative effect on FVIII:C activity, whereas increasing the amount of citrate added to heparin plasma significantly affected the FVIII:C recovery adversely (Table 3). Drawing

Table 3. Effect of calcium on the initial (CPD and heparin) factor VIII concentration (%).

Plasma type (n=6)	**Addition**	**% initial FVIII:C**	
		6 hours	**20 hours**
CPD plasma	none	74 ± 22	61 ± 11
	10 mM $CaCl_2$ + 2 U/ml heparin	–	81 ± 14
	20 mM + idem	–	83 ± 13
Heparin plasma	none	100 ± 20	78 ± 12
	10 mM citrate	–	70 ± 8
	20 mM citrate	–	55 ± 1
	10 mM CaCl2	–	96 ± 13

From: Smith JK, Evans DR. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

Table 4. The effect of lowering amounts of citrate on factor VIII recovery. Fibrinopeptide A (FpA) is measured to indicate activation.

Final plasma citrate (mM)	VIII:C at 22 h (IU/dl)	FpA (ng/ml)	Ca^{2+} (μM)	pH
20 (neat CPD)	68 ± 17	40	25	7.6
16	71 ± 13	30	36	7.7
12	80 ± 16	28	61	7.7
10	76 ± 20	25	77	7.7
8	86 ± 17	17	96	7.7
4	clot at 30′	13.350	276	7.8
Heparin	92 ± 22	23	955	7.9

From: Prowse CV. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

blood in serially lowering amounts of citrate (20 mmM–4 mM) shows that concentrations as low as 8 mM do recover substantially better amounts of FVIII:C without activation of the procoagulant activity as measured by fibrinopeptide A (Table 4). Heparin controls showed a stabilizing effect on FVIII:C over the time (Table 3) and a seemingly higher initial FVIII:C recovery (Table 4). This raises the question of what anticoagulant is preferred and what advantageous effects are to be expected from using heparin. Heparin could be used either as a primary anticoagulant or added to citrated plasma. In both conditions the precipitability of CIG and the recovery of FVIII:C are enhanced, an observation which was in principle described by Morrison in the 1940's [3]. The enhancing effects on the gel formation or cryoprecipitation permits a better CIG purification. On the other hand heparin is not acceptable as a primary anticoagulant for the cellular components and the remaining CIG poor plasma, because of its rapid loss of function. Mikaelsson et al [4] looked into the effect of Ca^{2+} on the molecular integrity and biological activity of the trace CIG FVIII:C at 37°C over a 30 hours time incubation (Figure 1a and 1b). CPD plasma with or without heparin behaved equal, showing a rapid loss of activity over the first 5 hours where heparin plasma showed a significantly less progressive decline in FVIII:C activity. Addition of $CaCl_2$ to a final concentration of 20 mM to heparinized CPD plasma (prevention from clot formation) remarkably stabilized the FVIII:C activity over the entire period of incubation. These experiments have been repeated under different conditions by amongst others Krachmalnikoff et al [5] showing the importance of the presence of optimal divalent cation concentrations in the medium for the recovery of specifically the trace CIG FVIII:C, where heparin plays a secondary role in enhancing the process of phase change.

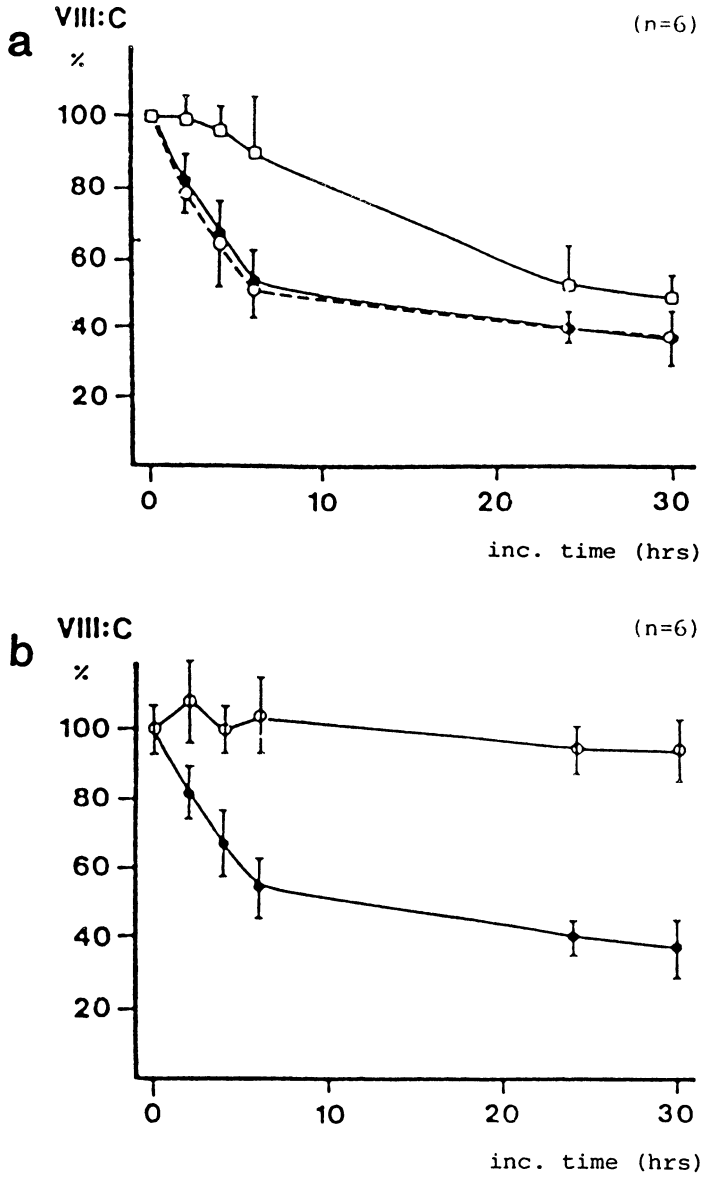


Figure 1. a. Stability of FVIII:C in CPD and heparin plasma at 37°C. b.Effect of Ca^{2+} on the stability of FVIII:C in plasma at 37°C. ○ = CPD plasma, ● = heparinized CPD plasma, ❑ = heparin plasma. From: Mikaelsson ME, et al. Blood 1983;62:1006–15.

The cooling process – Plasma when collected from the donor has to be cooled from 37°C. The first step in the processing is the separation from the cells by centrifugation, which usually results in cooling down to approximately 18–20°C in the core of the bags. The second step is the freezing process which needs specific attention. Here, the composition of the plasma determining the eutectic point which normally is around –27°C, the quality of the heat exchange accomplished by the way of applying the physical conditions of cooling and the area of exposure or thicknes of the layer of plasma are of paramount importance to achieve an optimal freezing process. The cooling can be done by different physical means, for instance alcohol/dry-ice, circulating supercooled N_2 or CO_2 gas or

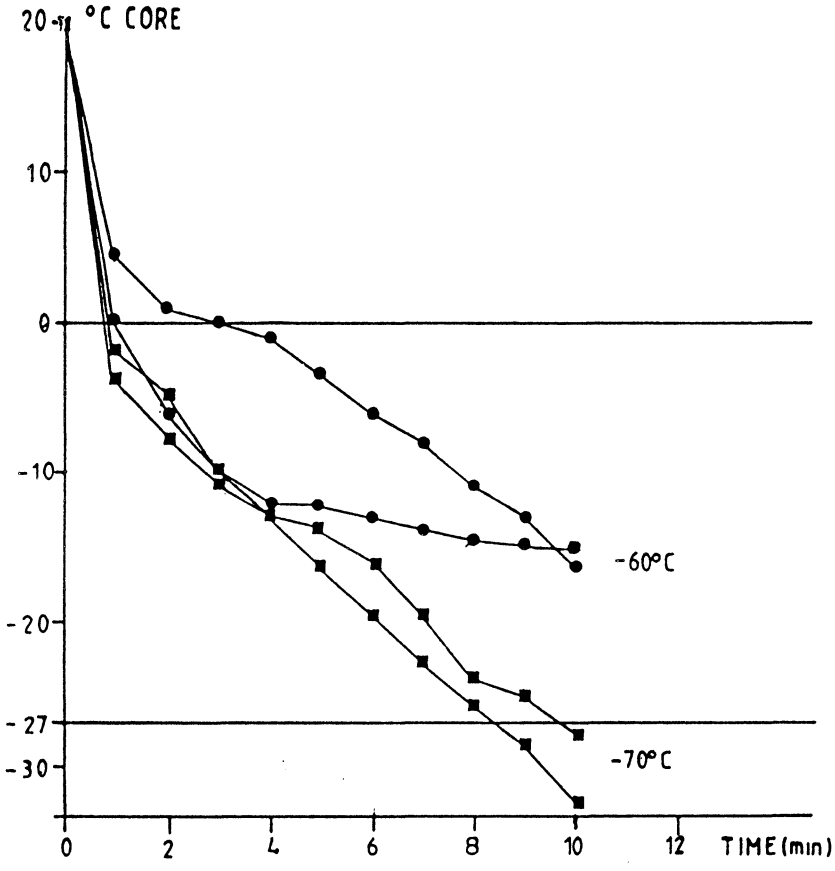


Figure 2. Effect of alcohol-dry ice starting temperature (● = –60°C, ■ = –70°C) on core temperature during plasma freezing (bags in horizontal position).

Table 5. Time of core freezing plasma in two thicknesses of layer (4 cm and 2 cm) and under fast and slow freezing conditions.

Freezing condition medium	Temperature	Thickness of plasma layers		
		4 cm	2 cm	
Alcohol dry ice	– 70°C	11 min	7 min	fast
Circulating N_2-gas	–100°C	12 min	10 min	
Stationary air	– 30°C	4 hrs	3 hrs	slow
Idem + insulation	– 30°C	19 hrs	15 hrs	

From: Over J et al. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

other means. Important are the temperature of the cooling environment during the freezing process and a constant exchange of temperature from the environment into the plasma and vice versa in which the surface area can contribute to optimize this exchange. Fiets and Feitsma [6] did an experiment where standard 450 ml bags filled with 250 ml of plasma were layered horizontally in a stainless steel meshwork basket and plunged into alcohol cooled with dry-ice to temperatures of respectively –60°C and –70°C. Temperatures were recorded continously through probes positioned in the cores of the bags. Additonal dry-ice induced a circulation by the escape of gas bubbles from the bottom of the alcohol container. Rapid cooling down to zero occurred, followed by a short period of supercooling and a slower freezing of the plasma. A constant temperature of –70°C made the core of the bags reach the eutectic point within 9 minutes (Figure 2). Similar experiments were done by amongst others Over et al [7]. They looked at the effect of different freezing conditions and different thicknesses of plasma layer on the recovery of the bulk CIG fibrinogen and the trace CIG FVIII:C (Table 5 and 6). Fast freezing was accomplished with either alcohol/dry-ice (–70°C) or cir-

Table 6. Effect of fast and slow freezing condition on FVIII:C, fibrinogen recovery and total protein.

Freezing condition medium	Temperature	FVIII:C IU/l	Fibrinogen g/l	Total protein g/l
Alcohol dry ice	– 70°C	467	10.5	23.2
Circulating N_2-gas	–100°C	513	11.2	23.7
Stationary air	– 30°C	490	13.0	29.5
Idem + insulation	– 30°C	433	14.6	34.9

From: Over J et al. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

culating N_2 gas (–100°C), where slow freezing was done with stationary air in a mechanical freezer (–30°C) or having the plasma bags positioned in an insulating polystyrene container in stationary air (–30°C). Temperatures were measured in the cores of the horizontally positioned plasma bags. In both conditions of slow and fast freezing a thin layer of plasma resulted in a shorter time needed for core freezing than a thicker plasma layer. The recovery of CIG's related to the total protein in the CIG fraction (fibrinogen to total protein ratio) was better when fast freezing conditions were applied: Total protein respectively 23.2 and 23.7 g/l versus 29.5 and 34.9 g/l reflecting the effect of moving corewards the plasma constituents when from the surface of the mass the water is frozen,

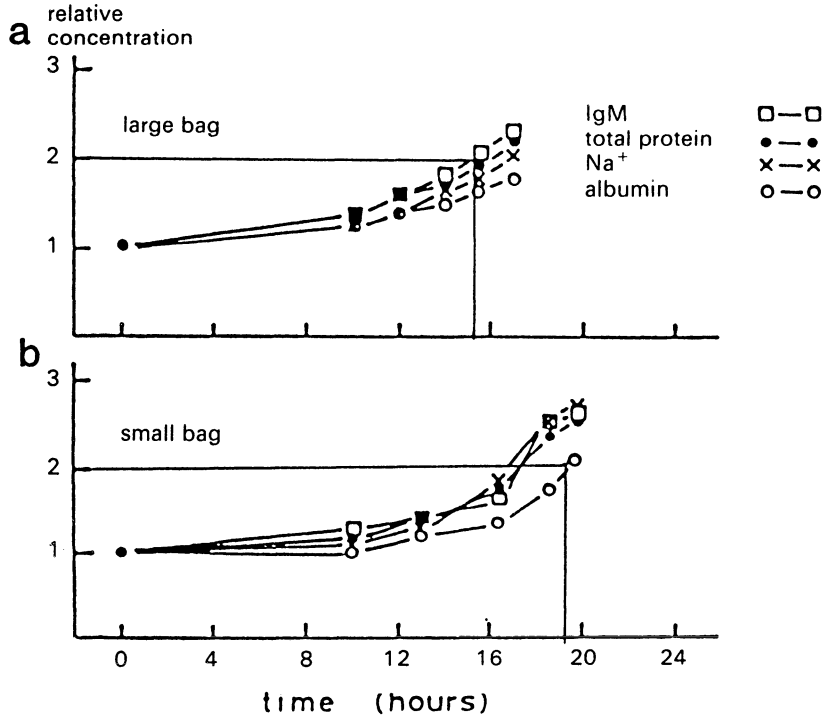


Figure 3. Core-accumulation of plasma constituents in plasma, frozen slowly (stationary air insulated –30°C) in thin (2 cm) and thick layer (4cm).
From: Over J, et al. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

changing slowly the freezing point of the mass. When in the time over both slow freezing procedures core concentrations of crystalloids and proteins are followed, the relative concentrations double or almost triple over the freezing period, affecting seriously the ionic strength, osmolarity and viscosity of the core mass (Figure 3a and 3b). These changes do effect the molecular structure and biological function of particularly the factorVIII/von Willebrand factor complex in the CIG fraction. So, it is recommended to keep the conditions of cooling and freezing as optimal as possible: pH and ionic strength of the medium, a fast cooling and freezing procedure in a thin layer of plasma to guarantee a rapid cooling to below the eutectic point to achieve a fast and solid core freezing.

Conditions of warming (thawing)

To separate the CIG from the remaining plasma protein mass warming of the frozen plasma under controlled conditions is done. Here, the same two areas of critical attention are to be distinguished: The medium and the warming process per sé (Table 7).

The medium – During the warming process ice crystals melt and will dissolve gradually the crystalloids, proteins and other constituents that belong to the solute fraction at low temperatures. This does indeed affect the pH and the ionic strength over the period of melting and may therefore further affect the molecular integrity and the biological function of the CIG's in the gel phase. The presence of agents enhancing the phase change process such as heparin and glycine are of importance to solidify the gel formed during cooling.

The warming process – Similar to the cooling process, warming or thawing is very much dependent on the rate of heat exchange, the temperatures applied and the control of the warming process. Time and determination of the desired endpoint to recover an optimal amount of CIG gel are of extreme importance as continuation of the thawing process passing 0°C may lead to a reverse change of phase back into the solute state. This implicitly means a loss of CIG in the supernatant. There are two major approaches in the thawing of frozen plasma in order to separate the CIG fraction:

Table 7. Conditions of heating (thawing).

Medium	pH ionic strength
Heating	rate heat exchange temperature control time determination of end point

Table 8. Time of thawing plasma as a batch or by thaw-siphoning under fast and slow thawing conditions.

Thawing conditions medium	Temperature	Technique of thawing plasma		
		batch	thaw-siphon	
Waterbath	20°C	15 min	12 min	fast
Waterbath	4°C	1 hr	45 min	
Stationary air	4°C	20 hrs	90 min	slow

From: Over J, et al. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

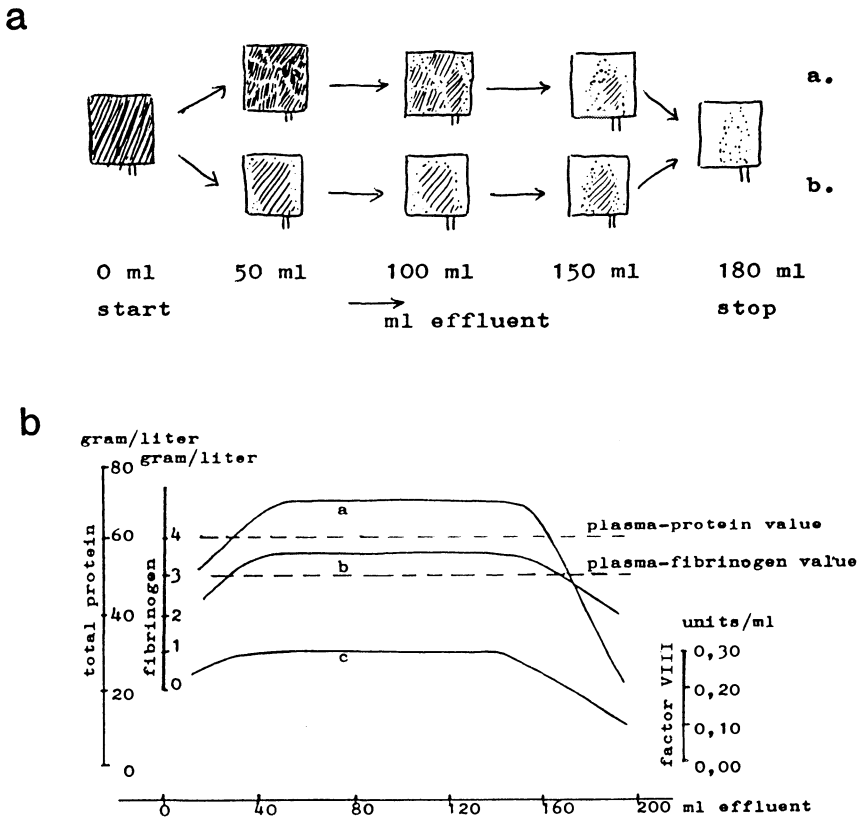


Figure 4. Effect of thaw-siphoning.

a. Depigmentation during thaw-siphoning, with (a) and without (b) tension.
b. Composition of the thaw-siphoning supernatant in consecutively collected samples; total protein (a), fibrinogen (b) and FVIII:C (c).

1. batch thawing, where solute and gel mix together over the melting process.
2. thaw-siphoning or continuous thawing, where in principle the solute is continuously separated from the gel during the melting process.

Evidently the warming can be done in a fast and slow way and at different temperatures of environment. Compared to the batch thawing, continuous approach is always faster irrespective of the temperatures applied. Over et al. have done experiments in which batch and thaw-siphoning of frozen plasma are compared when applying a waterbath at 20°C or 4°C, and stationary air at 4°C. The results clearly show the time effect of thaw-siphoning over the batch process (Table 8). Thaw-siphoning was first described by Mason in 1978 [8]; when thawing a bag of frozen plasma in a circulating waterbath at 4°C and allowing the melting solute to be siphoned off continuously, a much larger gel of CIG, a cryoprecipitate, was recovered. When analyzing this principle, melting of ice crystals seem to form capillary shaped channels through which the water and dissolved solutes are drained away, leaving the CIG gel behind (Figure 4a and 4b). The experiments done by Doorenbos in 1979 [9] show that under the conditions applied a certain amount of CIG (both fibrinogen and FVIII:C) redissolve from the gel phase into the sol phase over this process up to a certain point where obviously almost all water has been siphoned off, determining the endpoint of the separation process. Over et al [7] compared the effect of the different melting conditions of factor VIII recovery and specific activity, both for fast and slow frozen plasma (Figure 5a and 5b). Here, also the siphoning off of the melted solute in combination with a fast freezing process resulted in superior recoveries at very acceptable specific activities of this crude CIG mass cryoprecipitate. For large scale operations the principle has been adapted to a system of slow and gradual conditioning of the frozen plasma mass from –40°C to –10°C over a period of 5 hours, followed by a fast melting process under controlled conditions of warming (temperatures and exchange, Figure 6). Foster et al [10] demonstrated the ultimate superior effect of this approach on the recovery of FVIII:C in the CIG fraction from CPD plasma frozen within 6 hours following collection (Table 9).

Separation and purification of the different CIG's

When we combine the different aspects discussed, both in the cooling and the warming, the following principles could be included:

1. collection of plasma by plasmapheresis and immediate restoring in a closed multiple bag system of the calcium concentration using a solution of calcium lactate and heparin (Figure 7).
2. snap-freezing the plasma as a thin layer in the horizontal position in alcohol/dry-ice at –70°C.

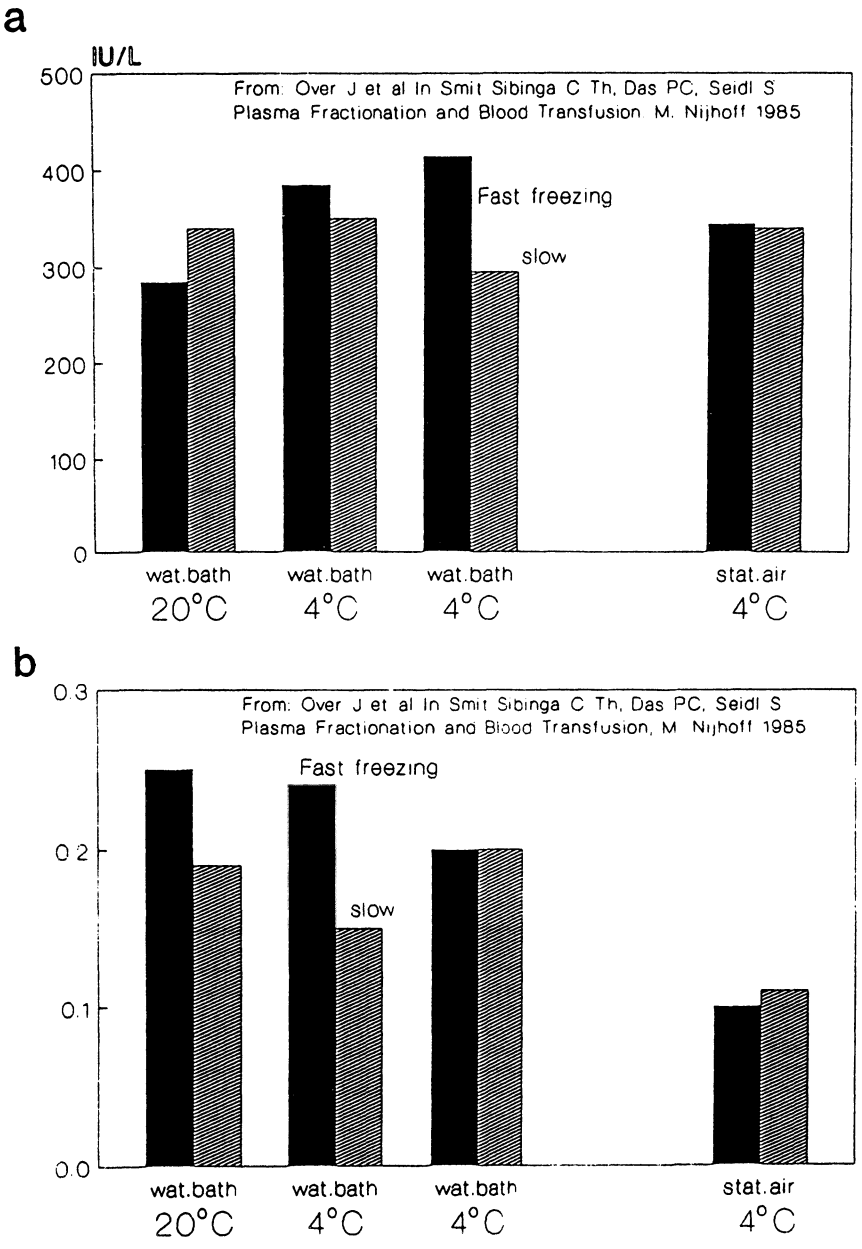


Figure 5. Thawing conditions of plasma.

a. Effect on FVIII:C recovery of different thawing conditions on fast and slow frozen plasma.

b. ffect on specific activity of recovered FVIII:C.

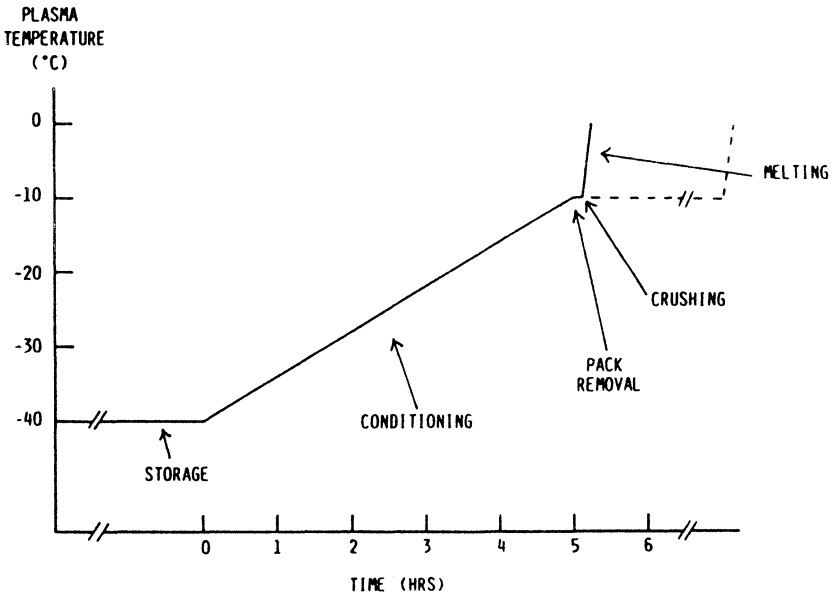


Figure 6. Schematic representation of the conditioning procedure for thawing batch plasma.
From: Foster PR, Dickson AJ. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

Table 9. Recovery of factor VIII from CPD plasma during continuous and batch thawing. Continuous thawing shows a significantly (s) better recovery, irrespective from cryoprecipitation 6 or 18 hours after collection.

6 hrs CPD plasma cryoprecipitate	**Continuous thaw (n=20)**	**Batch thaw (n=20)**
Weight (g/l plasma)	9.04 ± 0.64	8.49 ± 1.37
Protein (g/l plasma)	1.18 ± 0.10	1.06 ± 0.20
FVIII:C (IU/l plasma)	479.3 ± 101.1 s	316.5 ± 60.4
18 hrs CPD plasma cryoprecipitate	**Continuous thaw (n=12)**	**Batch thaw (n=12)**
Weight (g/l plasma)	9.53 ± 1084	8.93 ± 2.14
Protein (g/l plasma)	1.24 ± 0.17	0.88 ± 0.32
FVIII:C (IU/l plasma)	370.6 ± 66.8 s	278.2 ± 95.6

From: Foster PR, Dickson AJ. In: Plasma fractionation and blood transfusion. Smit Sibinga CTh, Das PC, Seidl S (eds). Martinus Nijhoff Publ. 1985.

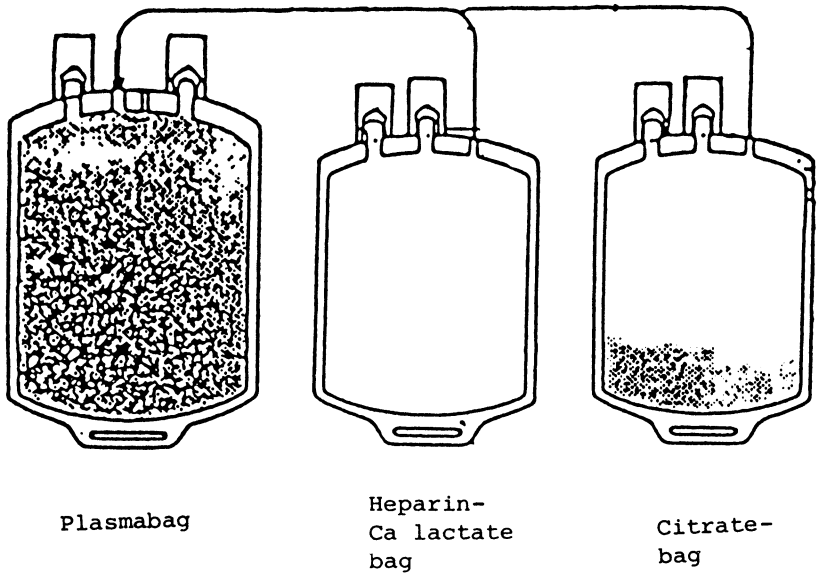


Figure 7. Plasmapheresis set (closed multiple bag system) for collection of citrated plasma (machine-apheresis) and conversion into heparin for CIG-purification.

3. warming at 4°C under the optimal conditions of heat exchange in a rocking waterbath and a carefully controlled timing. This allows recovery of an optimal mass of CIG, a first cryoprecipitate containing all CIG's.
4. redissolving this mass quickly at 37°C and addition of extra heparin to enhance a second phase change to allow separation of bulk CIG's from trace CIG's.
5. cooling to 0°C in a rocking waterbath allows a first separation of CIG fractions. This gel precipitate shows characteristics of an intermediate purity factor VIII/von Willebrand concentrate, but still contains a considerable amount of fibrinogen and also it has concentrated part of the enhancing factor heparin (Table 10).
6 redissolving this fraction completely at 37°C and adding calcium and a second phase change enhancing agent glycine, allows further separation and purification of CIG's.
7. setting the temperature for the phase change at 10°C in a rocking waterbath, allows the formation of a gel, rich in fibrinogen and fibronectin, leaving the trace CIG factor VIII/von Willebrand factor complex almost entirely in the sol phase.

Table 10. Comparison of characteristics of routinely produced heparin factor VIII preparations. Hep/CIG = double cold precipitation technique. Hep/CIG S = triple cold precipitation technique.

		Hep/CIG	Hep/CIG S
FVIII:C	IU/ml	18	22
Total protein	g/l	30	15
Fibrinogen	g/l	20	5
Heparin	IU/ml	7	0.5
pH 6.98 6.94			
Osmolarity	mosm/kg	350	300
Specific activity		0.6	1.5
Reconstruction time	min	10	3
Appearance		opalescent	clear

8. recovery by centrifugation at 10°C of the supernatant provides a purified factor VIII/von Willebrand factor concentrate stabilized by the extra amount of divalent cation and without the presence of glycine or heparin. This purified concentrate has the characteristics of a high purity factor VIII preparation at specific activities of 1.5 or more (Table 10).
9. the third cryoprecipitate can be used for the preparation of a purified fibronectin.

The overall loss of factor VIII activity following this procedure and applying the different principles is in the order of 65% as compared to conventional methodology, which usually recovers 20% at the best.

Conclusion

Plasma CIG's consist of bulk and trace proteins of which the factor VIII/von Willebrand factor complex is regarded to be clinically the most important. Each of the CIG's has its own specific temperature range and optimum for changing phase from sol to gel and back. The presence of enhancing agents such as heparin and glycine does enforce the gel formation, where pH and ionic strength together with presence of the divalent cation calcium at physiological levels stabilizes the trace CIG factor VIII/von Willebrand factor complex. This favourably affects the recovery of this specific CIG, while safeguarding the molecular integrity and biological function of the proteins. Finally, application of simple physical principles of cooling and warming under standardized conditions of heat exchange, time, pH and ionic strength allows separation and purification of CIG fractions, specifically factor VIII/von Willebrand factor complex and fibronectin at minimal loss of physiological activity.

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NEW SOLUTION FOR ORGAN PRESERVATION

J.H. Southard*

Introduction

Two methods are used to preserve organs for clinical transplantation: simple cold storage (CS) and continuous machine perfusion (MP). Simple cold storage uses a flushing solution to replace the blood in the organ and to cool the organ to about 4°C. Collins et al [1] first reported the successful 30-hour preservation of dog kidneys using CS and a preservation solution, Collins' solution, that contained a high concentration of K, Mg, PO_4, and glucose. Collins' solution was effective because it suppressed hypothermia-induced cell swelling. Machine perfusion continually perfuses the organ to keep it viable. The perfusate, at 4 to 10°C, is pumped through the renal artery at low pressure (40 to 60 mm Hg) and low flow (0.6 to 1.0 ml/[min.g]). This method was developed for clinical use by Belzer et al [2], who found that cryoprecipitated plasma provided a suitable perfusate for 3-day preservation. Both methods have been used clinically for about the past 20 years [3].

Neither CS nor MP was as effective for preserving livers, hearts, and pancreases as for preserving kidneys. Also, fewer attempts were made to transplant these other organs because of the poor success that was common before the development of cyclosporine immunosuppression. When cyclosporine became available for clinical use in 1984, the transplantation of organs other than kidneys increased throughout the world; one-year survival rates soon increased to near the 70% to 90% survival for transplanted kidneys. The major obstacle to transplanting these organs was the lack of a suitable method of preserving them for longer than 4 to 10 hours.

In this paper the development of a new organ preservation solution is discussed, that can preserve livers and pancreases for 2 to 3 days, and is equally effective for preserving kindeys. This solution, The University of Wisconsin UW solution, is currently used to clinically preserve these

* This work was supported by grants from the National Institutes of Health (DK 18624, DK 33554, and DK 35143).

three organs, but it has not yet been used for hearts. Recent experiments in our laboratory suggest, however, that the UW solution may also extend the duration of clinical heart preservation from the current limit of 4 to 6 hours to more than 24 hours.

Machine perfusion preservation

In 1967 Belzer et al [2] showed that cryoprecipitated plasma was a suitable perfusate for the 72-hour preservation of dog kidneys. The perfusate (plasma) was first frozen, then thawed and filtered to remove the cryoprecipitate that was composed of lipid material (lipoproteins) in the plasma. These unstable lipids formed aggregates that blocked the renal capillaries, increased pressure during continuous perfusion, and injured the organ. Continuous perfusion appeared to be the best way to preserve organs because the tissue is continuously supplied with nutrients (O_2, substrates, and other agents) and the organ's metabolic end products are continuously removed. On the basis of these findings, we continued trying to improve perfusion solutions and began examining the mechanisms by which hypothermia damages cells and organs.

Our initial efforts were directed toward finding the best colloid for MP. A colloid was necessary with MP to counteract the hydrostatic pressure generated by the pump and to prevent interstitial (extracellular) tissue edema. Other researchers reported that serum albumin was an effective colloid when used in a saline-based perfusion fluid [4,5]. We had trouble reproducing these results, presumably because of batch variations in serum albumin or because of differences in serum albumin from different sources. In 1980 we found that denatured albumin was toxic to kidneys during preservation, and we indicated that an improvement in the quality and duration of preservation would necessitate the use of other colloids [6]. Several synthetic and naturally occurring colloids were tested for kidney preservation [7]; the most promising was hydroxyethyl starch (HES), which has a relative molecular mass (M_r) of about 250,000 d. HES was modified by ultrafiltration against a 50,000-d cut-off membrane; this process removed the low-M_r components and contaminants introduced by the manufacturing process (ethylene oxide, etc.). Using the modified HES, we successfully preserved kidneys for 3 days [8]; kidneys preserved for 5 to 7 days appeared to suffer no vascular injury, although the recipient animals died.

This HES-containing perfusate was considerably different from the saline-based solutions used earlier. It contained gluconic acid (Na salt) as the major anion and chloride was replaced by gluconate, which suppressed hypothermia-induced cell swelling [9]. Cells swell at preservation temperatures (4 to 10°C) because Na-K ATPase (Na-pump) is suppressed and because the concentration of ATP is low [10]. In the absence of optimal Na-pump activity, Na enters the cell in exchange for K and decreases the membrane potential (i.e., it becomes negative inside

Table 1. Composition of preservation solutions.

	UW coldstorage	Machine perfusion
lactobionate	100 mmol/L	–
Na gluconate	–	100 mmol/L
Raffinose	30 mmol/L	–
KH_2PO_4	25 mmol/L	25 mmol/L
$MgSO_4$	5 mmol/L	5 mmol
Adenosine	5 mmol	5 mmol
Glutathione	3 mmol	3 mmol
Allopurinol	1 mmol	–
Insulin	40 U/L	40 U/L
Dexamethasone	8 mg/L	8 mg/L
Hydroxyethyl starch	50 g/L	50 g/L

pH = 7.4 (at room temperature); final Na+ = 30± 5 mmol/L (UW) or 130± 5 mmol/L (machine perfusion); final K^2 = 120± 5 mmol/L (UW) or 25± 5 mmol/L (machine perfusion); final mOsm/l = 320 mosm/l.

the cell). The lack of an effective membrane potential leads to an influx of chloride, additional Na, and water. Cells also swell because the concentration of colloidal (non-permeable) anions and proteins is higher in the cell than in the extracellular space [10]. This hypothermia-induced cell swelling is effectively suppressed by replacing chloride with higher M_r anions such as gluconate or lactobionate [9].

In addition to replacing chloride with gluconate, we also added agents to stimulate cell metabolism at hypothermia. Adenosine (10 mmol/L) and phosphate (25 mmol/L) were added to stimulate ATP production during hypothermic perfusion [11] and to improve kidney preservation [12]; glutathione was added as an antioxidant. This combination of agents (Table 1) provided a perfusate that was purely synthetic and that gave excellent 3-day kidney preservation [8] and clinical results [13]. But preservation with this solution was still limited to only 3-days.

Calcium was not an added component of our solution, and we had become increasingly interested in the role of this element in organ preservation. Our solution did contain 100 mmol/L of gluconate, which is a well known chelator of Ca. We therefore thought that gluconate might be removing Ca from the cell or from its structural components. The perfusion of tissues with a Ca-free medium (or a Ca chelator) disrupts the intercellular matrix and makes possible the isolation of individual cells, but an increase in the cytosolic concentration of Ca is cytotoxic [14]. Apparently we faced a paradox: too much Ca might be harmful, but too little might induce structural damage to the kidney.

We experimented with adding Ca to the perfusate and found an

optimum concentration for 5-day kidney preservation [15]. We also found an association between the Ca concentration and the preservation of mitochondrial function. Mitochondria isolated from kidneys that had been preserved in the absence of Ca for 5 days showed a loss of respiratory control; they also lost respiratory control if the perfusate contained 1.5 mmol/L of Ca. However, in the presence of 0.5 mmol/L of Ca (free Ca = 50 μmol/L), isolated mitochondria retained respiratory control. Sixty-three percent of kidneys preserved in the presence of 0.5 mmol/L Ca were viable, and all the dogs survived if they were pretreated with chlorpromazine and methylprednisolone. Calcium thus seemed an important factor in kidney perfusion, apparently maintaining the functional (and perhaps structural) integrity of cell membranes (mitochondria). We are currently using this newly developed glyconate- and HES-containing perfusion fluid in our clinic, as are others in their clinics, and the results have been excellent. The average duration of preservation in our center is about 26 hours; the maximum is more than 60 hours. The incidence of delayed graft function is 6%, and there has been very little primary nonfunction.

Most recently, we have used a modified version of the kidney perfusate to successfully preserve dog livers for 3 days with MP [16]. The modification was a reversal of the cation ratio. The kidney perfusate contained a high concentration of Na (about 130 mmol/L) and a lower concentration of K (about 25 mmol/L). This ratio did not prove suitable for perfusing livers. But after reversing the ratio of monovalent electrolytes (to 130 mmol/L of K and 25 mmol/L of Na) we consistently got successful liver preservation with the dog orthotopic transplant model.

Simple cold storage preservation

In 1984, when our center began transplanting livers and pancreases, the major cause of low success was poor preservation. We considered perfusion preservation the best method, but the urgent clinical need for liver and pancreas preservation warranted the study of CS. Jan Wahlberg, a visiting surgeon from Sweden, joined our laboratory and modified the kidney perfusate; the result was successful 3-day pancreas preservation [17]. The primary modification was the use of lactobionate (an impermeable anion that is lager than gluconate) and raffinose (an impermeable saccharide); the other ingredients were left unchanged (Table 1). Wahlberg showed that this solution effectively suppressed hypothermia-induced swelling in livers, pancreases, and kidneys [18]. Following Wahlberg, Jamieson and Sundberg used the same solution to successfully preserve dog livers for 48 hours [19], and Ploeg showed that the solution was effective for 3-day kidney preservation [20].

This solution, UW cold storage solution, is currently marketed by DuPont Pharmaceuticals under the trade name "Viaspan". It has been used clinically to preserve livers (up to 35 hours), kidneys (up to

49 hours), and pancreases (up to 29 hours) with excellent results [21,22]. Using this solution:

1. more organs can be transplanted,
2. the surgery can be a scheduled operation,
3. there is less delayed graft function, and
4. organs can be obtained from more distant locations and transported virtually throughout the world.

Mechanism of action of UW solution

The composition of the UW solution is based on our theory of how organs are damaged during hypothermic storage [23]. Because of the success of the UW solution, many centers have begun to examine its components to determine which are necessary for successful preservation. Our center is also investigating why the UW solution works in an attempt to further improve the solution.

Cell impermeants

As mentioned above, a primary problem in the hypothermic storage of organs is cell swelling, and the suppression of this swelling leads to moderately successful organ preservation. Solutions that have high concentrations of impermeant saccharides are somewhat successful for kidneys [24,25], but we found that the impermeant anions (lactobionate and gluconate) are the best agents for suppressing cell swelling in all organs studied. In addition, raffinose, phosphate, and hydroxyethyl starch are impermeants that contribute to the suppression of cell swelling.

Adenosine

Cells lose ATP when the organ is stored at hypothermia, and this ATP is degraded to adenosine, hypoxanthine, inosine, and xanthine. When the organ is reperfused, these purines are rapidly flushed from the cells and there is a deficiency of purine precursors for ATP resynthesis after transplantation [26]. Loading the cell with an ATP precursor (adenosine or adenine plus ribose) facilitates a more rapid and extensive regeneration of ATP. This effect was demonstrated in studies with isolated perfused kidneys [12], liver tissue slices [27], isolated hepatocytes [27], and renal tubules [28]. In renal tubules, the omission of adenosine from the UW solution not only leads to decreased ATP concentration when the organ is rewarmed and reoxygenated, but it also results in a considerable loss of cell viability [29]. We do not know if adenosine is necessary for the survival of whole organs after transplantation, but it is clear that ATP synthesis is stimulated and this should help the organ regain normal function after transplantation.

Glutathione

Much evidence suggests that damage induced by O_2-free radicals occurs after preservation and transplantation [30-32]. The origin of superoxide anions is not well understood; they could arise from the xanthine oxidase-catalyzed metabolism of hypoxanthine-xanthine, from mitochondrial reactions, or from other reactions in the cell or in circulating macrophages [reviewed in 33]. The cell contains a relatively high concentration of glutathione (GSH, 3 to 5 mmol/L), which has the single function of reducing hydrogen peroxide (generated from an iron-catalyzed reaction with hydroxylradicals) to water. The metabolism of hydrogen peroxide is catalyzed by glutathione peroxidase, which forms oxidized GSH (GSSG) and is converted back to GSH by glutathione reductase (NADH-requiring enzyme). Tissues lose GSH during cold storage or ischemia [34], and this loss causes the cells to be more sensitive to damage by O_2-free radicals [33].

We added GSH to the preservation solution to increase cellular defense against O_2-free radical damage. Many studies have demonstrated that the addition of GSH (or precursors of GSH) to cells increases their tolerance to ischemic injury [35-37]. We have also shown that GSH is an essential component of the UW solution. Renal tubules stored at hypothermia for 2 days in the UW solution without added GSH lose viability and are incapable of regenerating normal ATP concentrations [29]; with added GSH, the cells regenerate normal concentrations of ATP and remain fully viable after being rewarmed and reoxygenated. There is also evidence that stimulating the regeration of GSH protects stored hepatocytes [38]. Furthermore, we found that omitting GSH from the UW solution leads to a complete loss of the viability of dog livers stored for 2 days; by contrast, 80% of the animals survived when GSH was added to the solution. GSH also appears to be an essential component of the UW solution for preserving hearts [39].

Other agents

The role of insulin, dexamethasone, Mg, and allopurinol in the UW solution is unclear, and additional work will show whether these agents are essential. These agents have traditionally been used in organ preservation solutions, for reasons discussed elsewhere [23]. Briefly, allopurinol is added to inhibit xanthine oxidase and to suppress the formation of superoxide anions. Magnesium and dexamethasone are thought to provide increased membrane stability, and insulin facilitates metabolic reactions that may be necessary for organ preservation.

The UW solution appears to be a universal organ preservation solution that provides clinically useful preservation times for livers, kidneys, and pancreases. Although other solutions are effective for kidney preservation, they do not provide sufficiently long preservation for the other

organs. Despite the success of the UW solutions for livers, kidneys and pancreases, it has not proved successful for hearts tested in the large animal orthotopic transplant model.

Future developments in organ preservation

The goal of organ preservation research is preservation of unlimited duration. It may be that this end can only be achieved through cryopreservation (i.e., by freezing organs), and research in this field is progressing, albeit slowly. The prolongation of organ preservation to as long as 2 weeks may be possible with simpler hypothermic methods (above 0°C), but this achievement will demand a better understanding of the mechanisms by which preservation damages organs.

An increase in the duration of preservation to more than 1 week would 1) allow tissue matching for organs other than kidneys, 2) allow most organs to be transplanted in an appropriate donor virtually anywhere in the world, and 3) allow methods of altering organ immunogenicity to be studied in an attempt to reduce the incidence of graft rejection. Primary nonfunction of the liver and kidney is still a major problem. And the 4 to 10 hours of preservation now possible for the heart, lung, and intestine must be considerably extended to improve transplantation success for these organs.

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DISCUSSION

D.E. Pegg, J. Over

M. Harvey (Leiden, NL): Dr. Holme, we are interested in storing platelets which have been filtered directly after taking blood. Have you tried storage with your media with platelets that have been passed through a filter.

S. Holme (Norfolk, VA, USA): No, we have not done that yet.

C.Th. Smit Sibinga (Groningen, NL): Dr. Meyerstein, in your presentation about the neonatal red cells you showed that their ability to remain viable is substantially less than adult cells. Does that relate to the haemoglobin F? The second question is: Do you have any idea how long this status remains as the neonate matures. When does it disappear?

N. Meyerstein (Beer-Sheva, ISR): We followed up the ATP and DPG levels, degree of haemolysis and osmotic fragility during storage. We did not do any survival studies. In a previous study we tried to correlate the percentage of haemoglobin F in newborn cells with other parameters. When a baby is born, the blood contains 60-90% of haemoglobin F. We did not find any correlation between the amount of haemoglobin F present and any other parameters. It has also been shown that although purified foetal haemoglobin is more prone to denaturation than adult haemoglobin, there is no relationship between foetal haemoglobin content of the cell and the tendency to form Heinz bodies.[1] During the first two weeks of life, many of the red cells with high haemoglobin F concentrations are destroyed. So, I would believe that in two or three weeks after birth most of the special characteristics of the newborn red cell would have disappeared.

1) Nathan DG, Oski FA. Haematology of childhood and infancy. Third edition. Saunders CO 1987:25.

J.H. Southard (Madison, WI, USA): Dr. Meryman, two questions. It was very interesting to see that the alkaline interior as you propose, protects the red cell. Would a more alkaline exterior protect the red cells?

H.T. Meryman: Well, yes. There have been a couple of attempts in the past to maintain an elevated pH during storage by artificial means such as including an amberlyte ion exchanger in the bag. If you can maintain a high extracellular pH, then the intracellular pH is going to track it. If the extracellular medium has no penetrating anion then, when the chloride comes out, the only anion that can replace it is the hydroxyl ion, which results in an elevated intracellular pH. As you adjust the extracellular pH, the intracellular will track it. If we have a pH differential, that differential remains and the interior pH goes up parallel to the exterior. But the incredible thing that I find about this is that the pH discrepancy can be maintained for so long. In fact I suspect that the lactate that is being produced is paradoxically maintaining an intracellular elevated pH, because as the lactate ion diffuses out, it is going to be accompanied, as was the chloride, by OH^- influx.

J.H. Southard: The second question relates to the reperfusion injury. Is there such a thing as reperfusion injury in red cells once they are reexposed to oxygen?

H.T. Meryman: I am not aware that anyone has ever thought of that. I think your suggestion is unique.

J.T. Derksen: Dr. Southard, I noticed that you have adenosine in the preservation solution. Adenosine is a potent vasodilator, and can cause a drop in blood pressure. Did you ever consider replacing that with adenine?

J.H. Southard: No, we are working on that now in the cold storage solution and actually adenine seems to work reasonably well in our perfusion preservation solution. In fact, it is now a component of our perfusate, primarily because of a few reported cases here in Europe of bradyrhythmias caused by the solution. Actually the solution was used to flush the organs out with an additional solution just prior to transplanting them. So, the organ was filled then with adenosine and one reflow would start the adenosine to go into the system. Normally adenosine is metabolized when it is sitting in the organ and does not present a problem. But, because of those reports we will be working on that in the future.

H.T. Meryman: Dr. Southard, since you are dealing with a solution containing nothing but impermeate anions, do you see the intrcellular pH rise in your system as I do in the red cells.

J.H. Southard: Well, after hearing your talk, maybe in fact we are contributing to some successful preservation by a pH change. It is very possible that we do shrink the cells when we preserve them as whole organs. You did not see that in the tissue slices so much, but the livers shrink by 10-20% once stored in the UW solution. But we do not really know what the pH is. The only thing I can say is that we have taken hepatocytes and preserved them over a large range of pH's and then reflow them at normal pH. Those that were suspended in pH's greater than 7.6 or below 7.0 did not tolerate preservation well for that matter; pH 7.0 seemed to be an optimal pH, but that study was done in a poor reperfusion medium, as I showed here. However, when you reperfuse hepatocytes in a tissue culture medium you get an entirely different viability profile. So, I really cannot say how pH affects the viability until we repeat those experiments.

G.M. Fahy: Dr. Southard, in the beginning of your talk you mentioned quinacrine. I would like to know more about the rationale for its use.

J.H. Southard: The rationale of quinacrine, chlorpromazine and similar compounds of course, comes from the literature, studies on the breakdown of phospholipids in ischaemia. There are a couple of schools that believe that the loss of phospholipids is the key in ischaemic injury[1] and a number of papers have shown beneficial effects of things like chlorpromazine, trifluoperazine and quinacrine as suppressing phospholipid catabolism.[2] Well, this is a kind of a serendipendous type of approach, where you put in an agent that you think may be beneficial and try it. It sometimes is productive and sometimes is not. Currently, we are involved in measuring the rate of breakdown of phospholipids and preserve organs to see if these agents really are effective for that reason or not. Currently, we are involved in measuring the rate of breakdown of phospholipids and preserve organs to see if these agents really are effective for that reason or not. Most of these drugs have multiple effects, so it is hard to pinpoint whether or not it is just that like we do the right experiments.

G.M. Fahy: I would also like to know if you have looked at using glycine and cysteine instead of glutathione.

J.H. Southard: Yes, glycine and glutamate and a compound called thioproleine, which is a cyclic cysteine that is a complex to formaldehyde, and another oxothioproleine, which is metabolized to less noxious agents. Those combinations work as well as glutathione. We have not yet tried methionine, which has been suggested to me as a potential source.

1) Farber JL, Young EE. Biochem Biophys 1981;211:312-20.
2) Chein KR, Abrams J, Pfau RG, Farber JL. Am J Pathol 1977;88:539-53.

I think there are other glutathione delivery systems that are more stable than glutathione. I mean there is no evidence that glutathione either in the oxidized or in the reduced form gets into the cell, which makes your question why add it to a solution. What we do need is a better glutathione delivery system and some of these have been studied by Alton Meister including acetyl cysteine.[1] Some of these may be potent agents. We have looked at these and one of these precursors works as well as glutathione. Dithiotrietol does not protect for longer than 24 hours. It does protect at 24 hours, so I was suggesting that there may be a component involved in the change in the redox state of the protein sulfhydrol groups, that is a contributing factor to preservation injury.

C.Th. Smit Sibinga: Dr. Holme, I was intrigued by the last line in your first slide, where you said that by the extention of the storage of platelets in a particular medium, you might have the opportunity to sterilize platelets. What is it actually that you mean by sterilizing?

S. Holme (Norfolk, VA, USA): Ultraviolet radiation seems to be easier to apply with protein free media. This is something that was just suggested to me by a colleague but actually has not been done yet.

C.Th. Smit Sibinga: UV-light irradiation is not really sterilization, but trying to kill lymphocyte function. You have not gone that far to study the actual remaining haemostatic capacity of the platelets, because you showed only the recoveries with chromium or indium labelled platelets. However, the proof of the pudding is in the eating and that is in the real clinical efficacy of platelets, haemostasis. Any information about that?

S. Holme: No, we have not done any studies yet.

C.Th. Smit Sibinga: The point always is that when you collect human blood or maybe platelets by apheresis techniques, you collect a certain circulating population, so younger probably more active platelets, middle aged and older platelets. You put them together in a highly unphysiological environment – a bag and force them to survive as long as possible, although you try to make the conditions as optimal as possible. What type of cells is it actually that the major effects you noticed were on. Was it on the younger cells or was it more on the older cells?

1) Williamson JM, Boettcher B, Meister A. Prol Natl Acad Scie (USA) 1982;79:6246-9.

S. Holme: Well, we have not investigated that, it is difficult to say. Difficult to say also what kind of study can be done in order to investigate whether the old or young cells are better maintained during storage. Have you investigated this?

C.Th. Smit Sibinga: We looked very closely, besides all the metabolic parameters, into what the electronmicroscope morphology looked like. This provides at least an idea of how the different organels in the platelets do behave under these circumstances.

F.A. Ala (Birmingham, UK): I have a very small remark addressed to dr. Holme as a result of dr. Smit Sibinga's remark. Dr. Holme, when you mentioned sterilization, you meant psoralen-UV sterilization, which does more than murder the lymphocytes. That is not really what dr. Smit Sibinga was suggesting. It was really a sterilization which you were after and of course there is laser UV sterilization as well.

W.J. Armitage: Buffering capacity is clearly an important factor in these artificial media for storing both erythrocytes and platelets. Have any experiments been done using organic buffers such as tris? Is there any reason why these should not be used in such solutions?

O. Åkerblom (Stockholm, S): Tris was used in the beginning of the 60's in an experiment.[1] Tris was added at intervals during the storage to keep up the pH of the cell. This was quite successful, the DPG was kept high. However, as predicted by Sam Rapoport more than 50 years ago, at this high pH where DPG was well maintained, the ATP decreased rapidly.

C. Högman (Uppsala, S): I have some comments on the papers of Dr. Åkerblom and Dr. Meryman concerning the adenine nucleotides. Usually, we speak about ATP, and this is perfectly acceptable as long as we have physiological conditions because most of the adenine nucleotides are in the form of ATP which has the highest energy value. But during storage of red cells there is quite a change in energy charge. This means that an increased part of the adenine nucleotides are in the form of AMP and ADP. In my view it is more important for the red cell to maintain the total adenine nucleotides than the ATP. When the red cells enter into the circulation with a total adenine nucleotide pool which is unchanged, they can very easily phosphorylate up to a high energy level, reestablishing a high ATP and a lower ADP and AMP. But if they have lost adenine nucleotides so that the total pool is lower, they have great difficulties in regaining adenine nucleotides, because the concentrations of adenine

1) de Verdier C-H, Högman CF, Garby L, Kilander J. Acta Physiol Scand 1964;60:141-9.

and adenosine in the circulation are very low. I think that this is a very important factor. So, for instance after 5 or 6 weeks of storage there may well be strong decrease in ATP but you may have normal concentration of adenine nucleotides.

Concerning the morphology and maintenance of morphology, Dr. Åkerblom pointed out that these changes are reversible, at least to a certain extent. We found a few years ago that if you incubate the red cells *in vitro* so that you reestablish the normal morphology as much as you can and then relate the remaining changes to red cell survival *in vivo*, you get a very nice correlation.[1] That is why we should not speak about the morphological changes we see directly after storage, since most of these changes are readily reversible. But what cannot be reversed is important.

Dr. Meryman, I simply loved your results, so I congratulate you warmly. However, I think that it is not quite true that phosphate does not penetrate. In fact it does, but very slowly. Because of a breakdown of 2,3-diphosphoglycerate you will have an increase in the intracellular phosphate concentration and also, through penetration out of the cell, in the extracellular phosphate concentration.

O. Åkerblom: Phosphate penetrates the red cell, but slowly. The rate of penetration depends on the phosphate concentration, temperature, the pH and the concentration of monovalent ions like chloride.[2] The chloride seems to compete with phosphate for the fixed positive charges in the membrane system for transport of anions. If there is much chloride outside, phosphate will be transported in at a slower rate. Twenty-five years ago Beutler and Duron found that the addition of phosphate increased the ATP considerably for several weeks[3] and that might be because the Km value of the glyceraldehyde-3-phosphate-dehydrogenase is rather high. So, a small increase in phosphate will stimulate these steps of glycolysis and give more ATP. However, Beutler did not find a corresponding improvement of posttransfusion viability in eight weeks old cells.

H.T. Meryman: The solution that we settled on was limited by some very practical considerations. Part of my objectives was to create a solution that could be licensed. This means that the permissable concentrations of phosphate and citrate are limited. The solution we designed contains what we feel is the maximum acceptable concentration of both of these anions. There is also another limitation and that is in the total ionic con-

1) Högman CF, de Verdier C-H, Ericson Å, et al. Vox Sang 1985;48:257-68.
2) Deuticke B, Dierkesmann R, Bach R. In: Deutsch E, Gerlach E, Mozer K (eds). Stoffwechsel und Membranpermeabilität von Erythrocyten und Thrombocyten. Stutgart: Georg Thieme Verlag, 1968:430-40.
3) Beutler E, Duron O. Transfusion 1966;6:124-9.

centration. As Davson showed many years ago, if the ionic concentration falls much below 1 mmol of salt then there develops an increase in permeability of the red cell membrane. Our concentration of electrolyte is approximately 0.12 mmol. So, we are on the border line. I cannot increase the citrate anymore, so I have to put in the phosphate. It would be nice to have a solution that had twice as much citrate and just enough phosphate to support glycolysis. It is possible, as you point out, that this might maintain intracellular pH for a longer period of time and be beneficial, but we have these practical considerations that have been very confinding. Part of my pleasure with the results we have achieved is that they have actually been achieved within this very confining set of practical limitations.

D.E. Pegg: Dr. Meryman, as a matter of fact what is the volume of the red cells in your new solution during storage?

H.T. Meryman: The volume is roughly 80% of the lytic volume. The actual osmolarity is less than what would support the red cell. But this is compensated for by the fact that the chloride shift has reduced the volume of the cell, or rather reduced the amount of intracellular osmotic activity, so that the solution itself can be of a lower osmolarity than one would predict would be tolerated. Now, why swelling of the cells is beneficial I really do not know. I have a couple of theories none of which I think is correct. However, there is no question, in practice, but storing the cells in a hypotonic solution is beneficial.

O. Åkerblom: The addition of citrate to packed cells or washed cells is quite acceptable when just red cells are given. However, in the massive transfusion situation we have to give citrated plasma as well, and citrate added to packed or washed cells would increase the citrate load to the patient. This is one reason why there is no citrate in the SAG-M solution. However, how much citrate do we really need in the CPD solution? Plasma fractionators have told me we should reduce the citrate concentration. A good mixing of blood and preservative solution during the collection of the blood would allow a reduction to a 14 mmol/l or lower concentration. That would allow addition of citrate to the additive solution instead.

C.F. Högman: My comment is similar to Dr. Åkerblom's. We are just now doing experiments where we have reduced the citrate concentration in the anticoagulant. You have then two beneficial effects. One is the effect on the stability of factor VIII, which I think could be the major motive to make the change. The second one is to have in this way a reduction of the citrate load if you want to have citrate in the additive solution.

J.C. Bakker: Dr. Over, a question about the storage temperature of fresh frozen plasma. At the meeting at Aldershot Jane Needham reported that when plasma is frozen in liquid nitrogen and stored at close to liquid nitrogen temperatures (below –150°C), she could preserve the factor VIII levels up to five years.[1] She thought that was a superior temperature.

J. Over: I always have the impression that storage of frozen plasma at –30°C or lower would give the same results. So, I was a little bit surprised to hear this point. Speaking for plasma fractionation, I would not advocate the storage for such long time periods, because I really would like to process plasma as soon as possible.

C.Th. Smit Sibinga: I think Dr. Over is right. We have far from reached the luxury of being able to store plasma that long a time before actually processing it into the components needed in the clinic. The actual fact is that you need to preserve the plasma at least at or below the eutectic point. That is an important point of temperature, because some kind of melting process starts to occur if you go upwards in temperature, so up –27°C. However, in the still relatively coarse activity assay techniques we have specifically for factor VIII, it does not seem to come off significantly. One could question whether our assays are sensitive enough to really tell us what is happening if we store at different temperatures and what the differences really are.

J.C. Bakker: We really need good comparative studies and I thought she had a good comparative study, but the practical value may be limited. Dr. Åkerblom, I heard a presentation at a San Francisco meeting on blood substitutes.[2] A presenter had studied the hypoxanthine levels in stored erythrocytes and he saw a rise of hypoxanthine and had concern that upon transfusion hypoxanthine could contribute to a reperfusion-like phenomenon. Are you aware of more quantitative data on that and could that give rise to real concern for transfusion of stored erythrocytes?

O. Åkerblom: The hypoxanthine produced in stored red cells as a result of the breakdown of adenosine nucleotides, is very low quantitatively. A low adenine concentration does not influence the hypoxanthine very much. Hypoxanthine creates a problem when you incubate red cells with inosine and such substances, which are being used in a 10-20 mmol/l concentration. As shown by Spielmann et al in the 60's,[3] using their IAG

1) 50th Anniversary symposium of the Army Blood Supply Depot. Transfusion into the 21st Century. Aldershot, UK, October 4-5, 1989.
2) International Symposium on Red Cell Substitutes, San Francisco, May 16-19, 1989.
3) Fritzsche W, Siedentopf HG, Spielmann W. Klin Wochenschr 1965;43:881-7.

solution with a high concentration of inosine, transfusion of more than three units of blood gave a large increase of uric acid in the recipient. So that is why this was not a big success. Otherwise we have not seen any drawbacks with hypoxanthine.

R. Mitchell (Glasgow, UK): Dr. Smit Sibinga, you mentioned the question of thaw-siphon. A difficulty with thaw-siphon is, it is impossible to automate. Would you not therefore agree with Dr. Åkerblom, that we should be going for less citrate if calcium is all that important.

C.Th. Smit Sibinga: Well, you are quite right the thaw-siphoning technology as it was developed actually by the Australian Mr. Ernie Mason,[1] shows us a mechanism. Indeed, it is not a very practicable technique, but the crush-thawing is kind of thaw-siphoning which has been introduced. The matter of reducing the citrate indeed has an effect. Prowse has shown that also,[2] because you have not only a reversible type of binding of the cation, but also there is a substantial amount of calcium still remaining unbound in a lower citrate medium. That is certainly something that should be explored more intensely for the near future.

C. Högman: Dr. Holme, I think it is very important that we now construct suitable additive solutions also for platelets. I can just confirm that we have been using such a solution which is nothing very fancy. It is a citrate buffer with some sodium chloride, some phosphate and some mannitol in it and without any glucose, because the glucose is supplied by plasma. So, this medium is working together with plasma. We found then that the bicarbonate supplied with plasma was sufficient for 5 days of storage. As we indicated,[3] we have used this solution to suspend pooled buffycoats in. The medium for the platelets will then be something around 25-30% of plasma and the rest will be the additive solution. We have transfused somewhere around 2500 of these preparations into patients and we have made quite a lot of studies, also *in vitro* studies. It seems to work.

J. Over: Dr. Smit Sibinga, you made use of the stabilizing effect that heparin has on factor VIII in producing your concentrate at high recovery. But looking at your data, I got the impression that you were not able to reproduce the effect of a higher initial factor VIII level at time point 0, as has been described by Rock.[4]

1) Mason EC. Lancet 1978;ii:15-7.
2) Prowse CV. In: Smit Sibinga CTh, Das PC, Seidl S (eds). Plasma fractionation and blood transfusion. Boston: Martinus Nijhoff Publishers 1985:25-32.
3) Eriksson L, Högman CF, Johansson A. Abstract 20th Congress ISBT, London 1988, p.276.
4) Rock GA, Cruickshank WH, Tackaberry EX, Palmer AS. Thromb Res 1979;36:294-300.

C.Th. Smit Sibinga: To an extent that is correct. We could not consistently reproduce that initial elevation of factor VIII in the assay when drawing plasma in heparin as compared to what Rock did. There may be a difference in the assays and the standardization of the technique which might be responsible for the difference in our findings. However, on the other hand we found, and we still consistently find in our routine production, a significantly higher initial factor VIII content in the plasma as compared to standard citrated medium. So that is reproducible. On the other hand we have to admit that Rock has never gone into routine production. She has only produced the dedicated research figures and there is a difference as you know.

R. Mitchell: You also have to, of course, reverse the heparin before you do the assay, because it does interfere with the assay. That has always been a difficulty! The assay methods are quite different, the one she was using and the one you were using.

C.Th. Smit Sibinga: There are crucial differences, that is correct. However, progress has been made in neutralizing the heparin effect in plasma for factor VIII assay, allowing a more accurate measuring.

W.J. Armitage: Dr. Meryman, I have been trying to decide what is happening to the volume of these erythrocytes. You mentioned that the osmolality given was that of only the non-permeating ionic species. Is that correct? Presumably, the osmotic effect of glucose was not included, because it was assumed that the glucose permeated the cells. But under hypothermic conditions and with, if you like, an odd extracellular ionic composition, do you think that the transfer of glucose across the membrane might have been affected? Do you have evidence that the red cells were in fact fully equilibrated with the glucose?

H.T. Meryman: No, I do not have any evidence to that. I found it more fun to proceed without knowing what was going on.

M. Harvey: We transfuse very immature babies, and we are interested in data with respect to adenosine and nucleotide metabolites. Can anybody answer on the toxicity of nucleotide metabolites in this type of patient?

O. Åkerblom: It is not very likely that these comparatively small amounts of adenosine would give any marked toxic effects. But if you are very much concerned about that, you might try addition of inosine instead (in the same molar concentration) and a small amount of adenine to build up more ATP. Dr. Kreuger,[1] studying the toxicity of adenine in

1) Kreuger A. Transfusion 1976;16:249-52.

neonates, found that with a small amount of adenine present (0.12 mmol adenine per unit of blood), a small increase of the adenine concentration occurred in the plasma of the newborn during transfusion, and 20 minutes later, the adenine was all away. Obviously, most of the adenine given with the blood was transformed in the tissues into adenosine nucleotides. He stated that this low concentration of adenine gave no toxic effects. However, the higher concentration of adenine that we once used in ACD adenine blood and which is also present in the circle pack (new formula) and in ADSOL, may be risky in massive transfusion when more than 20 units of blood are given; the adenine present would be metabolized to dioxy-adenine, which can precipitate in the kidneys.

C.F. Högman: Dr. Kreuger's study was, if I recall correctly, not in very premature infants: They were mature infants. But in more recent days we have been using adenine containing red cells very extensively and for many years in premature infants. At least clinically there has been nothing indicating that this has caused any difficulty. I could further add that when we discussed this many years ago, we concluded that the premature infant also has very little xanthine oxidase. So, if you expect anything you would expect less of the 2,8-dihydroxyadenine formed in the prematures.

III. CRYOPRESERVATION ASPECTS

PLATELET FREEZING

P.C. Das, C.Th. Smit Sibinga

Bizzozero's description of platelets in 1882 certainly lagged behind by some 100 years to the morphological description of red cells derived from the discovery of van Leeuwenhoek's microscope. This historical analogy of slow progress seems to be valid today since platelets are more difficult to isolate and freeze than the red cells. This difficulty has been compounded since the demand for platelet transfusions has increased (Table 1) considerably in almost all countries including the USA, UK and the Netherlands [1,2]. This is partly due to the intensive support necessary to combat thrombocytopenia in oncology and leukemia patients and also due to transplantation and complex surgery. Routinely

Table 1. Number of platelets transfusion in idfferent countries.

USA	100×10^3 (1970)	100×10^4 (1987)
North London	40×10^3 (1977)	112×10^3 (1987)
Groningen	3.7×10^3 (1978)	9.8×10^3 (1987)

prepared platelets can be stored only for 3-5 days; this has certainly provided the impetus to freeze platelets for its subsequent clinical use.

Physiology

Following the release from megakaryocytes platelets circulate in the blood as cytoplasmic discs in a concentration of $200 \times 10^3/\mu l$. At any given moment of time the blood platelets are heterogeneous in nature as seen in normal platelet histogram. There are controversies regarding platelet size and age, although it seems that platelet density correlates with age in normal conditions [3].

About 70-80% of platelets circulate in the blood, the remainder is pooled in the spleen. The normal life span of platelets is about 7-11 days.

Table 2. In vitro tests for platelet functions.

Aggregation	collagen ADP adrenalin ristocetin
Hypotonic shock response (HSR)	
Clot retraction	
Morphology score	

Platelets are built with complex structure. Its surface membranes carry glycoproteins, provide receptors, allowing facilities to respond to the external stimulation. There are dense tubular and canalicular systems and microtubules maintaining the discoid shape. There are intercellular granula: α-granula secrete some of the platelet specific proteins (BTG, PF4); dens granula contain nucleotides, serotonin and calcium. The mitochondria are the measure site of energy production. The glycogen provides the basic food store and it exits in discrete particles in cytoplasma [4].

Functions

This small yet inticrate cell is capable of a variety of functions of which hemostatic plug formation is the main concern in platelet transfusion. This cell is shomewhat exitable, particularly at the site of damaged vessels. Here they would adhere, change shape stimulated by agonists like ADP, collagen and thrombin, and aggregate on collagen surface; and eventually undergo release reaction which would provide futher stimulants like ADP and thromboxane which is to amplify the process of hemostatic plug formation. This function would be inadequate or ineffective when there is:

1. failure of platelets to adhere to collagen;
2. failure to release ADP;

Table 3. Cryobiological effects on platelets.

Morphology	change
Enumeration	difficulty
Aggregation	reduction
Nucleotides	reduction
Granular contents	reduction
In vivo recovery	reduction (normal vs patient)
In vivo circulation	normal

3. failure to aggregate;
4. failure to make platelet phospholipid available for the clotting cascade.

In order to identify these detrimental effects generated by harvesting and futher manipulation of platelets a number of *in vitro* tests are available (Table 2). Although they are of considerable value in diagnosis of hemostatic defects in the patients, there is poor *in vivo* correlation following transfusion between these tests carried on donor platelets stored in liquid phase.

History of platelets freezing

The evolution and progress of platelet freezing have been reviewed [1,5].

Cellular injury during freezing is caused by transition of water to ice formed both extra and intracellularly. The rates of this injury would depend on first/slow freezing and subsequent thawing. However, the addition of cryoprotective agents can reduce the ice-crystal formation and lower the effective freezing point of the cell suspension. The addition of dimethylsulfoxide (DMSO) or glycerol are two such agents to lower the optimal cooling rate. Platelets cryopreserved with 5% DMSO are optimally cooled at 1-2°C/min while with glycerol at similar concentration, about 30°C/min is preferable. DMSO, however is toxic but fortunately, provides protection at low concentration.

The first attempt to cryopreserve platelets was tried without the addition of cryoprotectant [6]. Following thawing from –15°C/–30°C, it was transfused to a thrombocytopenic child; some evidence of transient hemostasis was recorded by this early work. The discovery that glycerol could protect sperm cells during freezing [7] led to a new era of cryobiology; this is being applied for platelets in 1958 and the improvement on this basic technique is still being continued [2,8]. Earlier studies used glycerol concentrations varying between 2-24%, accepting a 10-12% as optimum with slow freezing. But *in vivo* recovery varied between 20-50%. In order to improve *in vivo* results a study was conducted with DMSO in 1963 [9]. Since DMSO is toxic it must be used at low concentration with gradual addition of cells. In these studies 5-6% DMSO was generally used and freezing was carried out 2-4°C/min as controlled freezing or uncontrolled freezing. They were stored at –80°C in a freezer or in liquid N_2. Morphologically 50% of the platelets remain in discoid shapes. *In vivo* recovery of DMSO frozen platelets was interesting in the sense that in the healthy autologous situation 45% recovery was achieved [10] but in thrombocytopenic patients the recovery was lower [11]. The cryoinjury of the platelets did not seem to be uniform – half of the platelets were balooned and degranulated while the other half seemed to be structurally normal [12] The other problem (Table 3) faced following cryopreservation is on enumeration when platelet derived debris may be

counted as intact platelets [13]. There are other parameters that are affected by freezing: compared to fresh platelets, aggregation activity is reduced, and so is the ATP and ADP contents (about 70% to the fresh platelets). On the other hand, the recovered platelets circulate normally *in vivo.*

Methodological comparison

Our initial study comparing glycerol and DMSO has been carried out in volunteers [14]. Both methods followed a common pathway (Figure 1); 450 ml of blood was initially collected. Through platelets rich plasma (PRP) platelet concentrates (PC) were made by centrifugation. Platelet-poor plasma was used for making appropriate concentration of cryoprotectants, or stored for washing platelets required following thawing.

The platelet concentrate was transferred to a special freezing bag (Gambro) and cryopreserved with cryoprotectant solution. To equal volume of PC equal volume of 10% DMSO was added slowly while keeping the container cold. For the other system equal volume was added within 2 minutes. The bag was then put into a metal cassette and frozen at about 4°C/min (uncontrolled) and stored in the vapour phase of liquid N_2 for the DMSO system or frozen in the liquid N_2 and stored into the vapour phase of liquid N_2 for the glycerol system.

After thawing in a 37°C waterbath for 10 min, the DMSO was washed out with fresh plasma and platelets were resuspended in 50 ml of fresh plasma and allowed to stand for 1 hour at room temperature when final resuspension was done. In the glycerol system plasma was added stepwise at 15 minutes interval following thawing at 22°C for 10 min. The bag was centrifuged, supernatant plasma was removed leaving 50 ml of plasma; it was than allowed to rest for 1 hour at room temperature before finally suspended.

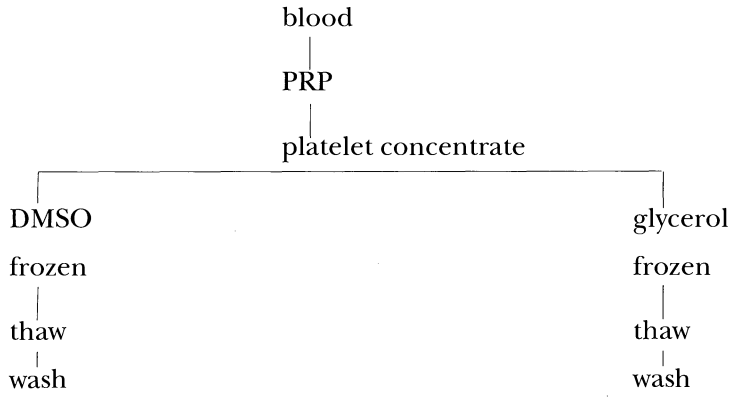

Figure 1. Comparison of two methods for cryopreservation.

Table 4. Platelet functions (%) in two cryopreservation systems.

	DMSO system			Glycerol system		
	mean	**(SD)**	**n**	**mean**	**(SD)**	**n**
Before freezing						
HSR	62.1	(10.9)	30	52.3	(11.1)	7
Aggregation	81.2	(12.1)	27	71.7	(20.6)	7
Clot retraction	66.3	(11.6)	26	63.5	(7.4)	7
After thawing						
HSR	20.6	(12.3)	20	3.9	(8.3)	8*
Aggregation	59.7	(12.5)	18	26.9	(25.6)	8*
Clot retraction	49.6	(16.1)	18	17.3	(7)	8*

* Significant difference ($p<0.05$).

For functional assessment 3 tests, (ristocetin aggregation, HSR and clot retraction at 3 hours) were chosen arbitrarily (Table 4). In view of the enumeration difficulty Coulter counter and microscopy was done in parallel and morphology was carried out by electron microscopy (EM). The recovery of platelets following freezing and thawing was about 70-80% in both the system. Under the EM 50% of the platelets are shown to be damaged. However, the functional parameters for frozen platelets show significant change in all 3 tests, while there was no significant change between glycerol and DMSO at the pre-freezing stage. Post-thawed DMSO platelets showed somewhat better results than that of glycerol.

Similar results were obtained when we extended this comparative study from random platelet donor to apheresis donated platelet concentrate derived from one single person. The ciruclating platelet life was analyzed by tagging ^{51}Cr to frozen platelets and reinfused autologously to 2 healthy volunteers. Results showed T1/2 life is about 3.8 days; this compared favourably with 8-10 days platelets life reported in the literature. There was no untoward reaction and the DMSO-induced garlic smell present initially disappeared completely within 24 hours from the volunteers.

Clinical: *in vitro* and *in vivo* study

Using DMSO-cryopreserved platelets we have undertaken clinical study in patients. All patients had received prednisone and antihistamine half an hour before the autologous platelets were transfused.

All platelet transfusions were given to patients prophylactically. Initial study has compared *in vitro* functional tests in eight consecutive patients who are treated with autologous bone marrow transplantation following

Table 5. Evaluation of frozen platelets for patients: *in vitro* tests.

	Before apheresis (sample A)	After apheresis (sample B)	After thawing (sample C)	C/B%
% ADP aggregation	78.7 ± 13 (56-100)	55.3 ± 31 (22-90)	21.4 ± 14.6 (0-49)	38.6%
% Collagen aggregation	79.2 ± 21 (28-100)	78 ± 16 (110-100)	28.1 ± 15.4 (14-60)	36%
% Ristocetin	82.2 ± 13.3 (56-100)	69.3 ± 13.7 (50-100)	50.9 ± 21.1 (17-83)	64%
% HSR	n.d.	70.8 ± 10 (57-95)	30.1 ± 13.1 (17-58)	43%

ablative chemotherapy for solid tumours [15]. From each patient a sample was collected before platelets were harvested by an apheresis machine, which were subsequently frozen according to our standard method. Following thawing and alliquotes were collected for bacteriology and for functional activity by ADP, collagen, ristocetin aggregation tests and hypotonic shock response. Results (Table 5) show while 80% platelets were recovered, the *in vitro* functional tests showed 40% loss of activity following freezing and thawing.

However, when they were transfused to patients there was no correlation between the *in vitro* functional tests, the individual platelet transfusion and the *in vivo* recovery. In this study a comparison is also available between frozen and autologous platelets and fresh (single donor) apheresis derived allogeneic donor platelets in six consecutive patients (Table 6).

In this prophylactic transfusion there was no bleeding episode seen in either of the series although better predicted recovery and corrected increment were observed in the series with fresh platelets. Melaragno et al [16] has done a similar clinical study comparing fresh versus frozen autologous platelets. In this study it was considered necessary to transfuse 2.5 times as many cryopreserved platelets to achieve a similar number of circulating platelets as produced by transfusion of fresh platelets. On the other hand it ought to be noted that leukemic patients' own platelets collected and frozen during remission, when given to the patient during thrombocytopenic stage, was as good as frozen HLA-matched platelets derived from normal donors [17,18]. Washing of platelets after thawing has been considered to cause platelet damage. Furthermore it is a time consuming procedure. Since our frozen platelets programme is oriented towards autologous ABMT patients who are supported for only a short thrombocytopenic period a clinical trial was carried out with DMSO or washed frozen platelets [19].

Table 6. In vivo results of (DMSO) frozen platelets (autologous).*

	Autologous (cryopreserved) n=5	**Allogeneic (fresh) n=5**
Amount of platelets transfused $\times 10^9$	237 ± 89 (123–430)	362 ± 85 (278–512)
Absolute 1 hr increment $\times 10^9$/l	13.6 ± 6.2 (6–25)	39.6 ± 24 (7–73)
1 hr corrected increment count $\times 10^9$/l	11 ± 6.5 (2.2–22.1)	19.8 ± 9.5 (4.1–30.9)
% Predicted recovery	46 ± 25 (8–100)	82 ± 40 (17.5–131)

*Results are expressed as mean ± SD (range).

Patients were thrombapheresed, averaging 2-5 platelet concentrates per patient. The concentrates were frozen and stored by usual methods. Each patient, however was transfused alternating with washed or not washed product. Results (Table 7) involving *in vivo* parameters of 42 transfusions in 12 patients indicate there was no significant difference in the percentage recovery or in corrected platelet increment. In addition one patient with leukemic relapse was transfused with his own platelets which were kept frozen for 4 years. After thawing the platelets had to be transported 100 km to a peripheral hospital.

Instead of immediate transfusion, this particular platelet concentrate, because of the logistics was transfused 3 hours after thawing. Results (Table 8) of four such transfusions show that both *in vivo* recovery and increment were very similar to our control trial study results. In addition we have treated an iso-immune thrombocytopenic newborn with DMSO frozen maternal platelets. After thawing and washing of DMSO with

Table 7. In vivo results of washed and unwashed cryopreserved platelets (42 transfusions in 12 patients).

	Without DMSO n=18	**With DMSO n=24**
Absolute platelets transfused $\times 10^9$	247 (144–573)	308 (90–645)*
1 hr increment $\times 10^9$/l	11.7 (2–24)	13.5 (–25)
1 hr corrected increment	9.2 (2.0–17.6)	8.7 (3.0–17.2)
% Predicted recovery	38 (9–72)	37 (11–87)

* Mean (range).

Table 8. A patient receiving 4 years old autologous frozen platelets (without washing DMSO and 100 km transport).

	n=4
Absolute platelets transfused $\times 10^9$	173 (162–207)*
1 hr increment $\times 10^9$	10 (6–14)
1 hr corrected increment	10.9 (6.9–15.9)
% Predicted recovery	50 (32–71)

* Mean (range)

Table 9. Platelet antibody detection using fresh vs frozen platelets derived from the same donor.

Week	Anti-PLA1 anti-body serum dilutions	Fresh platelets	MCHA ratio frozen platelets			
			LN2 (A)	–70°C (B)	LN2 (C)	–70°C (D)
1	1:160	2.36	2.02	2.36	2.44	2.25
2	1:160	2.12	2.04	–	2.55	–
6	1:160	2.01	2.11	–	2.25	–
9	1:160	2.24	1.75	–	–	2.16
21	1:160	1.85	–	1.57	–	1.62
23	1:160	1.85	–	1.85	2.20	–

Table 10. Advantage of frozen platelets in the laboratory.

- Convenience and reduction of time in assaying.
- Stable platelet target: comparison over time.
- Frozen selected panels: specificity of antibody (allo-, iso-, auto-).
- HLA phenotyped: crossmatching and donor selection.

fresh plasma the platelets were transfused showing good clinical effect with excellent one hour post infusion recovery. However, in multitransfused refractory patients we have given non-HLA matched frozen platelets with marginal results.

Necessity for freezing of platelets

Expertise, hard and soft-wares are now available for freezing platelets, although they are relatively costly. They could however be justified in selected areas:

1. autologously in selected patients such as in oncology. Despite poor recovery advantages are: no-immunization, no GvHD and no potential blood borne infection.
2. another potential area perhaps is to stockpile HLA-typed platelet concentrates for patients with refractory thrombocytopenia.
3. access to frozen platelet panels in the laboratory could expedite the platelet serological work and cross matching.

Use in the laboratory

Frozen platelets [20] when stored either in liquid N_2 or –70°C have been shown to retain their antigenic epitopes to detect and identify serum antibody and provide cross-matching possibilities. When tested in fluorescent-activated cell sorter method frozen platelets compared most favourably with fresh platelets obtained from the same individual (Table 9). The advantages of the use of frozen platelets in the laboratory, both immediate and potential, are summarized in Table 10.

Future trends

We have shown the value of frozen platelets. DMSO has been questioned for its toxicity but only in animal study this had proved to be of problem to the eye. In a longer study in man no such effect was found except transient nausea and smell of garlic [1]. Earlier clinical study has tended to create an ideal situation when fresh versus frozen platelets have been compared. Recently Valeri has claimed that *in vitro* loss of functions and *in vivo* recovery values of the frozen platelets are compatible to values of liquid platelet stored at room temperature for 5 days [1].

Frozen platelets have satisfactory haemostatic function immediately after transfusion whereas liquid room temperature stored platelets may remain dysfunctional 4-8 hours following transfusion. In the future further improvements of frozen platelets in their clinical application are to be expected. Amongst the technical advancements one interesting area is to be noted. The circulating platelets may be further fractionated on the basis of their size and volumes represented by different subpopulations (Figure 2). It has been shown [21] that the functional activities,

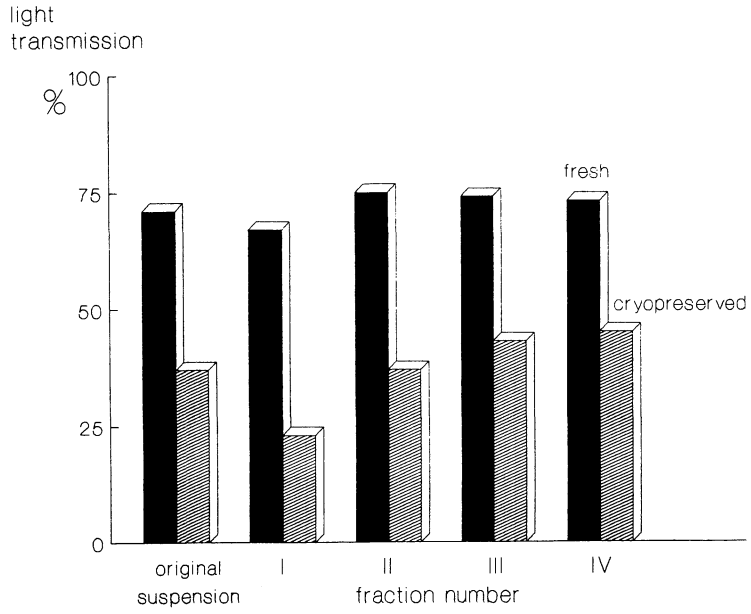

Figure 2. Volume distribution of blood platelets separated subpopulations (fractions 1-7). (Thomson et al 1982).

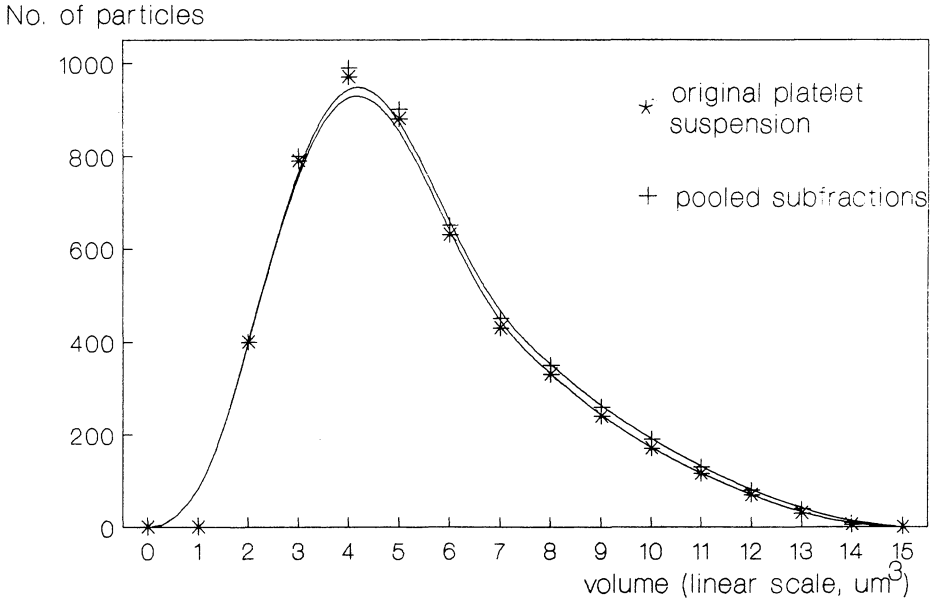

Figure 3. Platelet aggregation (collagen) and size dependence (fractions) in DMSO cryopreserved platelets (van Prooijen et al 1989).

granular contents, thromboxane-B production correlated well with the platelet volume distribution. Recent study [22] has shown that when these fractionated platelets are frozen separately, the larger platelets tend to retain better aggregability than that of the pooled platelet concentrate (Figure 3). It is possible that the membrane damage inflicted by cryopreservation procedure is more deleterious to the smaller cells than the larger platelets. The explanation for this may be due to membrane fluidity which is inversely proposional to platelet density – membrane anisotrophy being high in low density platelets [23]. Currently machinery and expertise are available in practise to harvest a selection of fractionated platelets based on volume/density. Such an approach can further improve the fate of cryopreserved platelets in clinical practise.

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PLATELET FREEZING: THE EFFECT OF SOME ADDITIVES

E. Richter

For platelet freezing we have followed groups who has used 5% dimethylsulphoxide (DMSO) for clinical purpose. Our results are very similar to those presented by Dr. Das. In autologous transfusion, the platelet survival is somewhat reduced in recovery when one compares with fresh allogenic platelets (Figure 1). However, the frozen platelets that survive after retransfusion seem to show normal circulating T1/2 life when compared with fresh platelets; the recovery of the latter was drastically reduced and T1/2 was much shorter when there was transfusion of mismatched platelets. As mentioned by Dr. Das, twice the amount of frozen platelets has to be transfused to achieve the levels obtained by fresh plate-

^{51}Cr-recovery and half-life (t1/2, d) of fresh and frozen platelets after autologous or allogeneic transfusions.

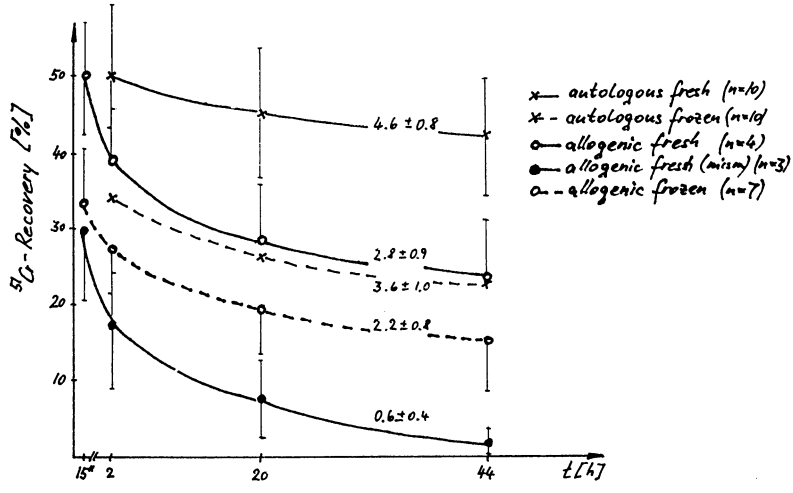

Figure 1. ^{51}Cr-recovery and half-life (t1/2, d) of fresh and frozen platelets after autologous or allogeneic transfusions.

Table 1. Corrected count increment (CCI) 1 and 20 hours after transfusion of fresh and cryopreserved platelets (mean ± SD).

	Platelet transfusion ($\times 10^{11}$)	1 hour	20 hours
Children			
PC_f (n=4)	1.9 ± 0.7	27.9 ± 19.7	20.2 ± 16.9
PC_c (n=3)	1.2 ± 0.7	23.2 ± 8.0	15.6 ± 12.7
Adults			
PC_f A (n=3)	1.4 ± 0.3	11.8 ± 3.7	5.2 ± 1.5
B (n=4)	1.5 ± 0.3	2.7 ± 2.2	1.7 ± 1.5
PC_c A (n=11)	1.2 ± 0.6	11.3 ± 5.3	4.4 ± 3.7
B (n=8)	1.0 ± 0.4	1.3 ± 1.2	1.1 ± 2.2

Table 2. Platelet counts and function after freezing and thawing (mean ± SE).

	Untreated control	Group	Glycerol	Me_2SO
Cell count (n=6)	3.80 ± 0.41	1	3.07 ± 0.29	3.35 ± 0.42
		2	3.06 ± 0.36	3.36 ± 0.39
		3	3.09 ± 0.36	3.47 ± 0.42
5HT uptake				
V_{max} (n=6)	19.03 ± 3.11	1	4.56 ± 0.65	7.86 ± 0.91
		2	6.07 ± 0.86	10.57 ± 0.89
		3	5.84 ± 0.89	10.14 ± 1.17
K_m (n=6)	0.96 ± 0.33	1	0.48 ± 0.05	0.62 ± 0.06
		2	0.53 ± 0.05	0.69 ± 0.05
		3	0.56 ± 0.06	0.72 ± 0.14
Hypotonic stress test (n=6)	75.5 ± 4.4	1	14.24 ± 1.42	38.58 ± 2.39
		2	15.42 ± 2.21	40.27 ± 3.83
		3	16.26 ± 2.03	44.08 ± 3.33
Aggregation				
Arachidonic acid (n=5)	86.3 ± 0.6	1	29.6 ± 3.2	60.6 ± 0.6
		2	39.5 ± 7.1	57.5 ± 3.9
		3	43.6 ± 9.6	59.0 ± 4.8
Collagen (n=5)	88.5 ± 0.8	1	26.8 ± 2.7	38.8 ± 4.7
		2	29.6 ± 3.8	43.8 ± 3.9
		3	25.6 ± 2.2	44.2 ± 4.5

Units: Cell count: cells$\times 10^8$/ml; V_{max}: pmol.10^8 cells.10^1 s; K_m: µmol/l; Hypotonic stress response: % reversal; Aggregation: maximum % change in transmittance.
Group 1 = treated control (no exposure to seleno-DL-methionine).
Group 2 = pre-freeze incubation with seleno-DL-methionine.
Group 3 = pre-freeze and post-thaw incubation with seleno-DL-methionine.

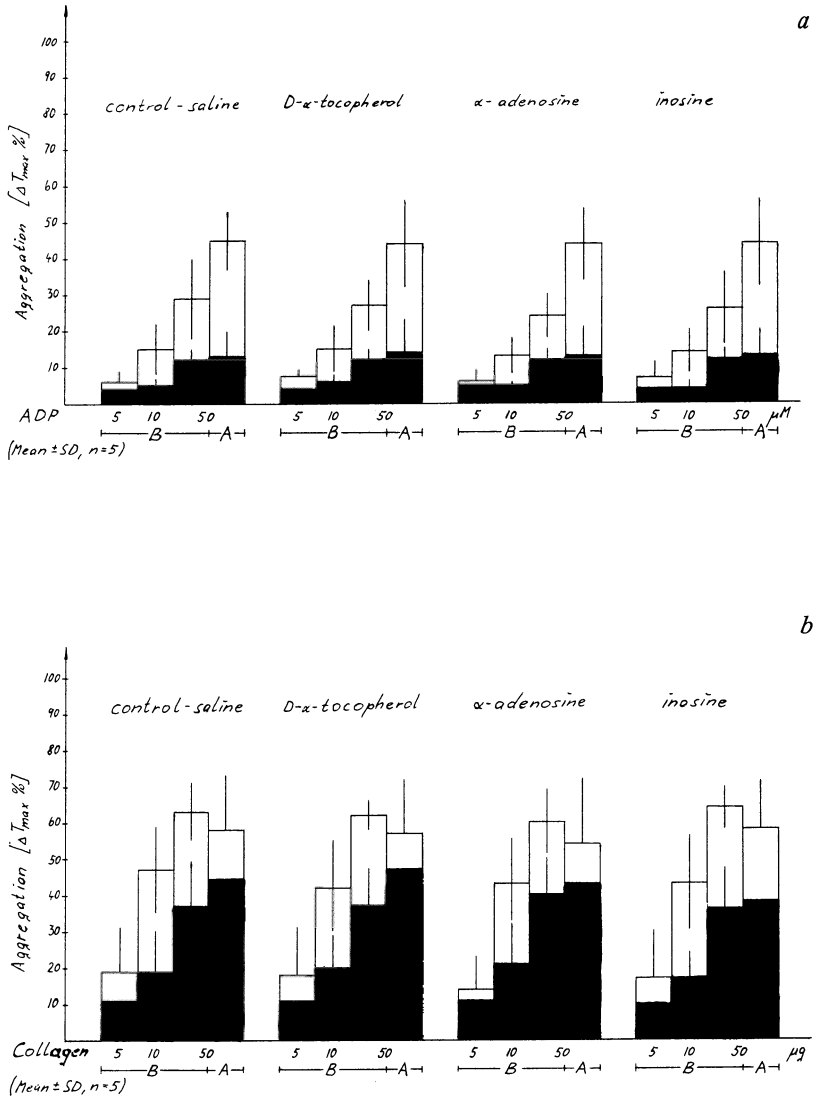

Figure 2a + b. Aggregation response of fresh (white) and frozen (black) platelets using various concentrations of ADP and collagen, and different additives.

lets. This may not sound to be good, but the bleeding problems of the patients are clinically resolved although the increment with the frozen platelets is always lower than that of the fresh platelets. Although twice the amount of frozen platelets are required in adults, the amount of frozen platelets available for children are enough for sufficient increase in count, as well as for sustaining $T_{1/2}$ life. In the adults, a clinical dose of available frozen platelets seems to be too low. Some of the problems that we have encountered in adult patients receiving cryopreserved platelets may be related to the immunological parameters (lymphocytotoxicity tests) as we have observed in cross-over study in patients receiving frozen and fresh platelets by measuring corrected increments at 1 or 20 hours (Table 1). Even when the cross-match is negative, one may find no increment in certain patients (B). However, sometimes one encounters patients who show poor recoveries over 2-3 transfusions, then 2-3 days later either HLA or sometimes platelet specific antibody is demonstrable in patients' sera.

It has been mentioned that conventional freezing will lead to 50-60% recovery after thawing, but there are some additives that might improve the recovery. These additives show beneficial effects on RBC and organ preservations in hypothermic conditions [1].

We have therefore done some experiments with additives like selenomethione in platelet cryopreservation along with Dr. Armitage in Cambridge [2]. We have used glycerol and DMSO along with the additives. With this system of cryopreservation we did not find any significant difference in HSR, 5HT uptake or aggregation response (collagen and arachidonic acid). Although there may have been slight improvement using glycerol as demonstrated by arachidonic acid aggregation, but experiments showed considerable variation as shown in the standard deviation of the results (Table. 2).

Afterwards we have followed α-tocopherol acetate addition as we could demonstrate that this is very effective in bone marrow cryopreservation [3]. In addition we have also added inosine and adenosine. There was no improvement with these additives as shown in the experiments when compared with the controls using saline (Figure 2). Only improvements could be demonstrated when the agonists such as ADP and collagen were used at 10-fold concentrations leading to reasonable aggregation. This only supports the remarks made by Law and Meryman [4] in 1985 that since the work of Djerassy [5] a lot of efforts have been made for improving the cryopreservation of platelets, but the improvement presently still remains around 50-60% recovery.

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INEXPENSIVE METHOD FOR CRYOPRESERVATION OF HUMAN PERIPHERAL BLOOD LYMPHOCYTES*

*O.W. van Assendelft, B.M. Jones, L. Stoddard III, T. Kelly

Liquid water is essential to the structure and function of living cells and it is thus not surprising that solidification of water by freezing is usually lethal to the cells. Paradoxically, however, appropriate freezing can also preserve cells for long periods of time in a viable state. The challenge to cells during freezing is not their ability to endure storage at very low temperatures, rather it is the lethality of the temperature zone from approximately –15°C to –60°C that the cell must traverse twice: once during cooling and once during warming.

At low temperature, e.g. at liquid nitrogen (N_2) temperature (–196°C), no thermally driven reactions occur in aqueous systems; liquid water does not exist below –130°C, only crystalline or glassy states exist. In both these states the viscosity is so high that there is no diffusion over finite time span. The only reactions that can occur in frozen aqueous systems are photophysical ones such as formation of free radicals and the production of breaks in macromolecules as a result of a direct hit by ionizing or cosmic radiation.

The chief physical events occurring in cells during freezing are the following. Down to about –5°C cells and the surrounding medium remain unfrozen because of supercooling and of freezing point depression by protecting solutes. Between –5°C and –15°C ice forms in the external medium but the cell contents remain unfrozen, albeit supercooled. The intracellular, supercooled water has a higher chemical potential than that of the water in the partly frozen extracellular solution. In response to this potential difference, water flows from the cell to freeze outside the cell. If cooling is sufficiently slow, the cell is able to lose water rapidly enough to concentrate the intracellular solutes, to eliminate supercooling, and to maintain the chemical potential of the intracellular water in equilibrium with that of the extracellular water. Thus, the cell de-

* The use of trade names is for identification only and does not constitute en-dorsement by the Public Health Service of the U.S. Department of Health and Human Services.

hydrates but the cell contents do not freeze. If the cell cools too rapidly, it is not able to lose water fast enough to maintain chemical potential equilibrium, becomes supercooled, and eventually freezes intracellularly. Available evidence indicates that when about 90% of cell water is removed, the residual 10% will not freeze at any temperature [1]. This paper describes an inexpensive method of processing human peripheral blood mononuclear cells for storage in the vapor phase of liquid N_2.

Lymphocytes

Viable lymphocytes are used in a variety of assays for monitoring cell-mediated immunity. Frozen, viable lymphocytes have been used in mixed lymphocyte culture response to various mitogens [2-5], in cytotoxicity assays [6-8] in tissue typing [9-11] for cell surface marker studies [12,13] for culture of virus from human immunodeficiency virus-infected individuals, and as quality control material for flow-cytometric phenotyping [14,15]. Preservation of viable lymphocytes is particularly useful because it allows batched determinations with cells, enables immunological studies to be performed under fixed and optimal experimental conditions, and allows retrospective studies of cells obtained sequentially, using new or improved assays as they are developed. Although various methods for the cryopreservation of lymphocytes have been described in the literature [11,16-22] there is no standardized method and no one method has gained general acceptance. In general, mononuclear cells are harvested with a density gradient technique from freshly drawn, anticoagulated whole blood. The cells are suspended in a cryopreservative solution containing a tissue culture medium, bovine or human serum, and dimethyl sulfoxide (DMSO) in a concentration of 8–10% [23-25]. The use of glycerol instead of DMSO is not recommended [23,26]. Freezing is performed at a controlled rate of 1-2°C/min down to about –30°C, then 3-5°C/min down to about –80°C [27]. Finally, the frozen cells are stored in liquid N_2 (–130°C to –170°C, depending on the proximity to the liquid phase). This process is relatively time-consuming and the equipment required for freezing at controlled rates is expensive. We have used an inexpensive method, the "double book bag method" for more than a decade with fully satisfactory results.

Isolation of mononuclear cells

Mononuclear cells (normal blood: 80–85% lymphocytes, 15-20% monocytes) are separated from sodium heparin-anticoagulated blood with a Ficoll-based density gradient, density 1.077-1.080 g/ml at 20°C. Although cell separation is recommended within 6 hours of drawing the blood acceptable results are obtained if the specimens are processed within 24 hours [12]. Heparin salts or ACD as anticoagulant give better separation results than do EDTA salts [12].

1. Transfer 15 ml to 20 ml aliquots of heparinized blood to 50 ml conical bottom, polypropylene, screwcap centrifuge tubes (e.g. Opticul™, Becton Dickinson, Lincoln Park, NJ); dilute each tube to 40 ml with phosphate buffered saline (PBS; 0.15 mol/l, pH 7.2; 8 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, and 200 mg KH_2PO_4 per liter) and mix well.
2. Inject 10 ml lymphocyte separation medium (LSM™, Organon Teknika, Durham, NC) slowly onto the bottom of the tubes with a sterile syringe and a blunt-tipped, 18 gauge cannula.
3. Using safety cannisters, centrifuge the tubes at room temperature for 20 min at RCF = 800× g. Allow the centrifuge to come to a halt without using the brake.
4. Aspirate the supernatant plasma and PBS carefully by vacuum to about 5 mm above the layer of mononuclear cells. Do not disturb the interface.
5. Transfer the mononuclear cell layer with a sterile Pasteur pipette to a clean, sterile 50-ml centrifuge tube. Add PBS to a total volume of 50 ml, mix by inversion and centrifuge the tubes in safety cannisters for 15 min at room temperature at RCF = 200× g.
6. Carefully decant the supernatant from the cell pellet and mix the pellet thoroughly with a vortex mixer. Where appropriate, combine the cell pellets from a single individual in a 50-ml centrifuge tube and dilute to 30 ml with PBS for a final washing.
7. Centrifuge the cell suspension at room temperature for 10 min at RCF = 200× g. Decant the supernatant carefully, mix the pellet with a vortex mixer and add 5 ml PBS to the suspension.
8. With a singel channel cell counter (e.g., Coulter™ Hemo-W or ZBI) count hte number of cells per ml. The cells will be stored at a concentration of 1×10^7 cells per ml.

Storing mononuclear cells

Reagents

1. Dimethyl sulfoxide (DSMO; molecular biology grade) 20% (v/v) in RPMI-1640 tissue culture medium (Gibco, Grand Island, NY) with penicillin and streptomycin added. The reagent is prepared fresh each day and kept on crushed ice until required. RPMI-1640 (liquid, cat. # 320-1875) contains D-glucose, reduced glutathione, phenol red, inorganic salts ($Ca(NO_3)_2$, KCl, $MgSO_4$, NaCl, $NaHCO_3$, Na_2HPO_4) and amino acids [20].
2 Fetal calf serum (FCS; Hazleton, Lenexa, KS), inactivated at 56°C for 30 min. The inactivated serum is stored at –20°C in volumes for approximate daily use. New lots of FCS must be inspected for the absence of particulate matter.
3. Penicillin/streptomycin solution containing 10,000 units penicillin G and 10,000 μ g streptomycin per ml; 5 ml of this solution is added to 500 ml RPMI-1640.

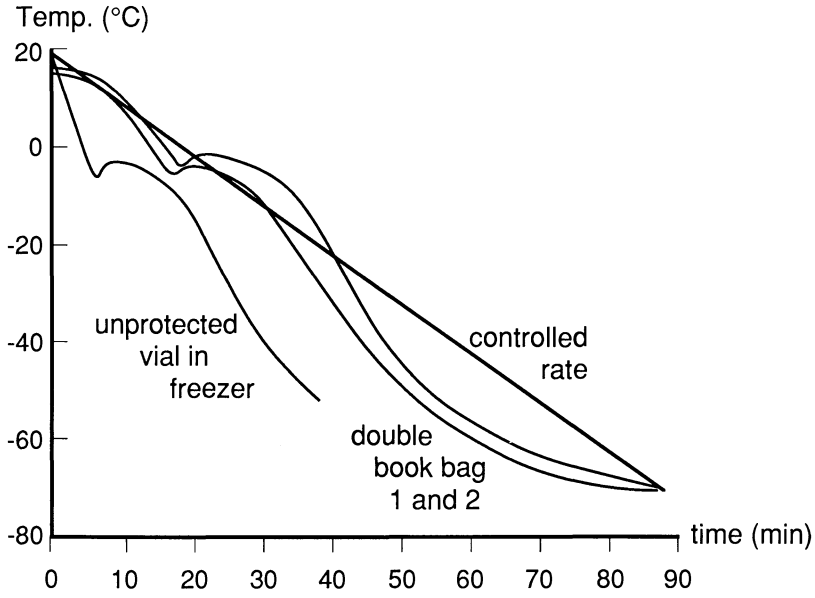

Figure 1. Temperature decrease over time in vials with 1 ml aliquots of mononuclear cells. "Unprotected" and "double book bag" vials in –75°C chest type freezer. "Controlled rate" represents a temperature decrease of 1°C/min.

Procedure

1. Label the required number of 2-ml polypropylene screwcap vials (Intermed Nunc 48× 12.5 mm cryotube) and pre-cool at –20°C.
2. Dilute the suspension of mononuclear cells to 30 ml with PBS. Centrifuge for 10 min at room temp at RCF = 200× g.
3. Carefully decant the supernatant and mix the cell pellet thoroughly with a vortex mixer. Resuspend the cells in FCS to a cell concentration of 2×10^7 cells per ml. Pre-cool the suspension on crushed ice.
4 Slowly, drop by drop, with continuous swirling, add an equal volume of DMSO/RPMI-1640 (20/80%, v/v) to the FCS/cell suspension
5. Dispense 1-ml aliquots of the cell suspension into pre-cooled screwcap vials; each vial will contain 1×10^7 cells.
6. Wrap the vials from each individual in a paper towel and place the wrapped vials in a pre-cooled (–20°C), 26.5× 41 cm padded envelope (Jiffy Packaging, # 5). The "book bag" can contain up to 50 vials. Fold the envelope in half, tape shut, and place in a second, larger (37×50 cm) padded envelope (Jiffy Packaging, # 7). Also fold and tape this outside envelope.

7. Place the "double book bag" overnight in a –70°C freezer. After 12 to 24 hours transfer the vials to 133× 133× 51 mm cardboard boxes (capacity 81 2-ml vials) and place in the vapor phase of a liquid N_2 freezer (e.g. 120 cm diameter, MVE Cryogenics, New Praque, MN). The decrease of temperature over time was recorded with a thermistor temperature probe (Omega Digicator, 400 BIA-F) inside vials of cell suspensions in "book bags" in a –70°C freezer. Figure 1 shows the results of two separate, representative experiments.

The temperature decreases at a rate of about 1°C/min for the first 20 min, plateaus briefly around –5°C for about 5 min, then steadily decreases at a rate of about 1.5°C/min. For comparison, a line representing controlled temperature decrease of 1°C/min is also shown.

Thawing of frozen cells

Thawing liquid N_2-stored mononuclear cells is the final critical step to ensure viability of lymphocytes to be tested. Good results are obtained when the following procedure is followed meticulously.

1. Remove vial(s) from freezer and place in a 37°C waterbath in a biological safety cabinet (See universal precautions). Swirl and agitate vials gently until just before the last ice crystal melts. The average time for this step is 3 min.
2. Remove vial(s) from waterbath and place on crushed ice.
3. At room temperature, transfer contents of the vial(s) to a 50-ml centrifuge tube with a Pasteur pipette. Slowly, drop by drop, add 10 ml of a FCS(20%)/RPMI-1640(80%) mixture; mix well by gently swirling.
4. Centrifuge, at room temperature, for 10 min at RCF = 200× g.
5. Carefully decant the supernatant and resuspend the cells in either FCS(20%)/RPMI-1640(80%) or FCS(20%)/PBS(80%). The amount of diluent added depends on the final cell concentration required.

Universal precautions

To address concerns regarding transmission of human immunodeficiency virus (HIV) the Centers for Disease Control developed the strategy of "universal blood and body fluid precautions" in 1985. The concept stresses that all patients should be assumed to be infected for HIV and other blood-borne pathogens [28]. In separating mononuclear cells from blood, appropriate precautions and safety measures must be taken. All processing of blood specimens is performed in a biological safety cabinet; personnel are required to wear gloves and disposable gowns; centrifugation is carried out with capped tubes in cannisters to prevent aerosol formation.

Cell recovery and cell viability

Because of cell losses during washing and pipetting, recovery of cells after freezing/thawing is 60-65%. Some improvement can be obtained by carefully rinsing centrifuge tubes and pipettes with diluent when transferring cell suspensions from one container to another.

Viability of thawed frozen cells has been tested using the trypan blue exclusion technique [29]; 95% of recovered cells are viable when either a controlled rate freezing process or the "double book bag method" is used. In contrast with others [30], no selective loss of viable lymphocyte subsets could be demonstrated [12]. Recovery of viable cells decreases rapidly when mononuclear cells are harvested from blood stored for more than 24 hours.

Conclusion

The "double book bag method" is an inexpensive and effective means for cryopreserving mononuclear cells. An advantage of the method is that cells can be frozen as soon as they are separated from blood, thus minimizing the time cells are exposed to possible cytotoxic effects of liquid DMSO [31]. The recovery of cells, 60-65%, is acceptable; the viability of recovered cells, 95%, is excellent. Investigation of the method has shown that fetal calf serum has good cryoprotective properties and that a freezing rate of 1-1.5°C/min gives good results.

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CRYOPRESERVATION OF HUMAN STEM CELLS

F. Beaujean

Autologous bone marrow transplantation (AMBT) is widely used to circumvent the myelotoxicity of intensive therapeutic regimens. Numerous clinical results provide evidence that autografting limits myelosuppression [1] and/or reconstitutes the host's hemopoietic system after lethal marrow injury [2]. Studies in recent years have demonstrated that recovery of hemopoiesis can also be achieved by the infusion of peripheral blood stem cells [3]. Whatever the source of stem cells, i.e. bone marrow or peripheral blood, they usually have to be stored for long periods before transplantation. The success of engraftment depends on the dose and the functional integrity of the cells infused, i.e. the quality of the stem cell collection and the storage procedure. Since the initial observations of Barnes and Loutit in rodents, studies in a variety of animal species [4,5,6] have evaluated low-temperature storage of marrow cells for functional use. Following these data, the efficiency of human stem cell cryopreservation has been intensively documented [7,8,9].

Recent developments in the field of ABMT have resulted in increasing manipulations before freezing. Numerous procedures are currently used either to concentrate stem cells or specifically to remove residual tumor cells from the suspension. However, the results of cryopreservation must be seen in the light of such procedures. This paper summarizes the principal developments in this area, reviews the available methods for stem cell preservation which yield the best results and discusses some of the problems currently unsolved in the transplantation of cryopreserved stem cells.

Collection and processing of human stem cells

Techniques for the harvest of bone marrow (BM) or peripheral blood stem cells (PBSC) have been described elsewhere in detail. When stem cells are to be frozen, a special effort must be made to maximize the number of cells collected. Actually, cell losses resulting from the freezing process and from manipulations before freezing must be taken into account. Approximately 2 to 3×10^8 nucleated marrow cells/kg of body

weight are collected to ensure engraftment after cryopreserved autologous bone marrow transplantation (ABMT). Large numbers (4 to 5×10^8 marrow cells/kg) are indicated when *ex-vivo* manipulations are used to purge marrow from tumor cells. With regard to PBSC collected by leukapheresis, it is difficult to assess the precise dose of mononuclear cells necessary to obtain engraftment. This point will be discussed later.

Developments in hemopoietic colony stimulating factors (CSF) have resulted in interesting novel applications for stem cell collection [10]. A recent report [11] described the use of granulocyte-macrophage colony stimulating factor (GM-CSF) after chemoterapy to increase the number of circulating hemopoietic stem cells and thus improve the yield of blood cell collection. The application of such a procedure to bone marrow collection might be of interest. However, further studies are required to evaluate the ability of such "liberated" progenitors to ensure stable engraftment following cryopreservation.

The quality of the feezing process depends on a number of factors:
a. the composition of the cell suspension to be stored;
b. *ex-vivo* manipulations before storage; c) freezing procedure.

Both bone marrow and circulating stem cells can be cryopreserved without separation, using a simple and clinically-proven procedure. However, while the freezing process promotes the survival of stem cells, differentiated cells (granulocytes, red cells) which contaminate the suspension are lyzed. As a result, clumping may occur, leading to loss of stem cells. Recently, significant progress has been made in stem cell processing prior to freezing. In addition to the main goal of enhancing the performance of cryopreservation through the removal of granulocytes

Table 1. Isolation of mononuclear cells from bone marrow by density gradient. (Cobe 2991) (n=50)

	After collection	**After separation**	**Final recovery (5%)**
Nucleated cells $\times10^8$/kg	2.71 ± 0.77	0.63 ± 0.30	23.2
Mononucleated cells $\times10^8$/kg	0.83 ± 0.35	0.56 ± 0.37	67.5
CFU-GM $\times10^4$/kg	8.51 ± 4.0	7.2 ± 3.8	84.6
Cell contamination			
RBC: Hematocrit	28 ± 5	0.1	0.1
Granulocytes: % NC	67 ± 7	13 ± 12	1.9

Mean ± SD (NC: nucleated cells)

and red cells, the various methods of stem cell processing present other advantages:

1. reducing the volume of the suspension, with consequent savings of storage space;
2. allowing further ex-vivo manipulation of the graft.

Most methods involve blood cell processors and are safe, reproducible and far less time-consuming than manual techniques. There are two main approaches, concentration and/or isolation of mononuclear cells. Cell concentration is achieved by sedimentation and/or centrifugation using the Cobe 2991, Haemonetics H30, V50. The mononuclear cell fraction is separated either by centrifugation (Cobe 2997 or Dideco) or by the use of a density gradient in a blood cell processor (Cobe 2991, Haemonetics V50) [12,13]. In our institute, such processing prior to storage is now performed almost systematically using a technique adapted from that published by Gilmore et al [14]. It associates the use of a Ficoll Hypaque density gradient in the Cobe 2991 blood cell processor. The results obtained with this procedure are given in Table 1. As indicated, a minimal loss of hemopoietic progenitors is observed. Moreover, clinical investigations clearly demonstrate that this technique does not impair the engraftment of such treated and cryopreserved cells.

The widespread use of ABMT has encouraged the development of various methods for removing residual tumor cells from stem cells. These techniques are applied before freezing and may have adverse effects on post-cryopreservation stem cell viability. Lopez et al [15] using a chemotherapeutic agent (ASTA Z) to purge bone marrow ex-vivo, showed that the remaining myeloid progenitors were more sensitive to freezing. In our experience, particularly poor post-freezing stem cell recoveries are observed when bone marrow is treated with monoclonal antibodies reactive against Calla antigen and complement. In contrast, treatment with anti-T cell antibodies does not affect recovery. Stoppa et al [16] studying the feasibility of cryopreservation of marrow cells previously grown in culture, reported major losses of both nucleated cells and hemopoietic progenitors.

The final cell concentration of the suspension, whether or not it is processed, remains an important freezing parameter. Best results are obtained with cell concentrations of less than 5×10^7 cells/ml.

Cryopreservation

In order to maximize stem cell viability, cryopreservation must comply with certain rules which have proven clinical implications.

For human stem cell cryopreservation, the most widely used cryoprotectant is demethylsulfoxide (DMSO) which has been shown to be superior to glycerol [17]. DMSO rapidly penetrates the cells, thus avoiding prolonged periods of equilibration. When DMSO is used alone,

optimal results are obtained with a 10% final concentration [9,18]. Several authors have shown that the detailed procedure of DMSO adjunction is crucial to cell viability. Since the report of Ragab [18], it has been recommended to add DMSO slowly, diluted in a solution containing serum proteins which have an additional cryoprotective effect. Most teams use a freezing solution containing 20% DMSO diluted either in tissue culture medium (RPMO–TC199) often supplemented with 10 to 15% of heterologous serum or in autologous plasma. As DMSO concentrations higher than 1% are toxic to stem cells at temperatures above 0°C, an equal volume of the solution is added to the stem cell suspension on ice. To simplify the procedure, we recently started using a 4% solution of human serum albumin (routinely used in clinical transfusion), to dissolve DMSO. In our experience, cell viability, function and recovery is similar to that observed with other freezing solutions (Table 2). The freezing process must be started as soon as possible after a short equilibration period between the cell suspension and cryoprotectant. In effect, even at 4°C, Douay [19] reported a loss of 25% of hemopoietic progenitors after 5 min of contact with DMSO and over 75% cell loss after 30 min. Likewise, after thawing, any manipulation of cells before infusion must be performed as quickly as possible.

In recent years, Stiff [20] has investigated a freezing procedure using a mixture of DMSO and an extracellular cryoprotectant, hydroxyethyl starch (HES), with final concentrations of 5% and 6%, respectively. In addition, freezing is performed without a rate-controlled freezing apparatus, simply by placing the suspension in a –80°C freezer. The DMSO/HES mixture was found to improve the recovery of differentiated granulocytes, thus reducing macroscopic clumping after thawing. This technique presents a particular interest for the freezing of unfractionated bone marrow. In addition it is simple and does not require

Table 2. Cryopreserved bone marrow: *in vitro* results after freeze-thaw-wash procedure. (Ficoll Hypaque isolated cells)

Freezing solutions	**n**	**% Cell recovery**	**% CFU-GM recovery**
20% DMSO 10% Human serum 70% TCM (washing with saline/plasma)	20	79 ± 18.7	64.4 ± 3.5
20% DMSO 80% HSA (4%) (washing with saline/2% HSA)	32	69.9 ± 14.6	63.2 ± 37.3

Human serum albumin 4% (Bio-Transfusion).

expensive equipment. Stem cell recovery and clinical results are satisfactory [21].

A strong relationship exists between the viability of cryopreserved stem cells and the freezing rate (generally controlled by programmable freezers). Using 10% DMSO, numerous studies have shown that the optimal freezing rate ranges between 1 and 3°C/min from 0°C to –50°C. Below –50°C, a rapid freezing rate (5°C/min) may be used until –150°C. During the freezing process, the transition of the cell suspension from liquid to solid phase should be as short as possible. Particular attention is paid to the freezing rate after the phase change. Abrams et al [22] reported poor cell recovery and survival using a cooling rate exceeding 3°C/min after the phase change. Similar observations were made by Gorin [23]. Storage below –150°C is recommended. Recently, Bandini [24] reported good recovery following cryopreservation at –196°C for more than 5 years. Different types of cell containers may be used (plastic bags or vials) with similar results.

Thawing should be rapid, preferably in a 40°C water-bath. The thawed marrow is infused either immediately or after a dilution-washing procedure. Animal studies [4] have shown that the rapid infusion of undiluted marrow may result in an osmotic shock due to the high tonicity of the cryoprotectant.

Stepwise dilution of the cell suspension after thawing significantly increases stem cell recovery. The use of such a procedure has the advantage (especially with unseparated bone marrow) of removing simultaneously DMSO, lysed granulocytes and the products of hemolysis. The major problem with this approach is the presence of macroscopic clumping after centrifugation, resulting in significant cell loss. Numerous authors have reported the infusion of unwashed stem cell suspensions with no toxicity. However, when peripheral blood stem cells are used, particularly large volumes have to be infused after thawing. In this situation, post-thaw washing and centrifugation reduces the volume of the suspension. We systematically wash cryopreserved marrows or circulating stem cells in a stepwise fashion [25] with a saline solution containing 2% human serum albumin.

Quality control of the cryopreservation procedure

Numerous indirect laboratory assays may be used to evaluate the efficiency of the freezing process. The first parameter to examine is cell recovery. As we have seen, large losses of differentiated cells can occur due to freezing, and thus the recovery of mononucleated cells (i.e. the fraction containing stem cells) is the most relevant. In our experience, more than 80% of BM or PB mononucleated cells are recovered after thawing and washing.

Since human pluripotent stem cells cannot be enumerated, functional viability is determined in terms of committed hemopoietic pro-

genitors including granulo-macrophagic, erythroblastic procursors (CFU-GM – BFU-E) and multipotent mixed progenitors (CFU-GEMM). Studies of cryopreserved stem cells indicate recoveries of 54 to 110% for CFU–GM [7,26,27], 64 to 85% for BFU-E [8] and between 54 and 90% for CFU-GEMM [8,26]. Values are similar for BM and PB stem cells. However, particular attention must be paid to culture conditions. For example, when the source of colony-stimulating activity (CSA) in the cryopreserved CFU-GM cultures is human placental conditioned medium, α-thioglycerol appears to stimulate growth and optimize plating efficiency. Since α-thioglycerol is not necessary when fresh cells are plated, it can be postulated that cell damage during cryopreservation renders CFU-GM hyporesponsive to this source of CSA [28]. Few data on the recovery of megakaryocytic progenitor cells (CFU-MK) are available. Recently, Berthier et al [29] reported between 70 and 80% of CFU-MK recovery after freezing when appropriate culture conditions were used.

The minimal number of cryopreserved marrow cells and CFU-GM required for engraftment in man is dificult to establish, but more than 1×10^8 marrow cells per kg seem necessary for ABMT [30]. Concerning the dose of CFU-GM to be infused, the results are much less clear. Many authors recommend 5 to 10×10^4 CFU-GM per kg, but successful grafts have been described with lower doses [31]. In some studies [32], the rapidity of myeloid hemopoietic recovery post-graft has been directly correlated to the number of CFU-GM infused, although this is not the case in our experience [33]. In the above conditions, the majority of ABMT are successful. In the rare cases where engraftment is difficult to obtain, one of the following factors may be involved:

a. the diagnosis (hematologic reconstitution is generally longer in leukaemic patients than in patients with solid tumors);
b. previous chemotherapy (possible effect on the medullary homing of the graft).

The number of circulating stem cells necessary to achieve engraftment is still controversial. Some studies have shown complete hematologic reconstitution with an infused dose of CFU-GM from between 2.2 to 9.4×10^4/kg [3,34]. In contrast, incomplete engraftment and even failure has been reported [35] with higher doses (above 30×10^4/kg). Such discrepancies may be related to the different protocols used to collect circulating stem cells. Acutally, different ratios of CFU-GM/pluripotent stem cells may be obtained. Further studies are needed to resolve this question.

In conclusion, autologous cryopreserved stem cell transplantation has considerably improved the treatment of malignant diseases, by allowing the use of higher doses of chemotherapy and/or radiotherapy. Techniques of stem cell cryopreservation are efficient, simple and standardized, making them easy to apply in most blood banks. Nevertheless,

the continued efficacy of cryopreservation must be confirmed with the advent of new cell processing methods.

Acknowledgements

We thank Mrs M. Sigal for expert secreterial assistance and D. Young for help with the english translation.

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PRESERVATION AND STORAGE OF HUMAN BLOOD PLASMA PROTEINS

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Introduction

Human blood plasma has been the primary source for a number of therapeutic transfusion products for more than forty years now. This situation will continue for quite some time, notwithstanding the arrival in the future of preparations produced by recombinant-DNA technology. Nowadays, a variety of plasma proteins is needed for substitution therapy. These include coagulation factors (among others factors VIII, IX, VII, von Willebrand factor), protease inhibitors (antithrombin III, Cl-esterase inhibitor and, under investigation α_1-antitrypsin), immunoglobulins (both normal immunoglobulin and specific immunoglobulins, derived from selected plasma containing high-titer antibodies directed against a specific pathogen) and albumin preparations, the latter being the longest existing plasma product. Other plasma componnents may be needed in the near future like activated factor VII and activated protein C.

For making the best possible use of the limited supply of human plasma several precautions have to be taken. Optimal preservation of the proteins of interest starts as early as the blood is collected from the donor, and continues to be a major concern all the way during processing to the final product. In general, the efforts are directed to preventing denaturation of the proteins (the therapeutic as well as contaminating ones) and avoiding activation of the complex protein systems of proenzymes, cofactors and their inhibitors. Also proteolytic degradation of the proteins and bacterial growth should be minimized. Other objectives, like extent of purification and eradication of the viral risk associated with the use of plasma products, may interfere with the aim of avoiding denaturation. In such a conflicting situation one should look for the best compromise.

Application of low temperatures in plasma fractionation has provided some effective solutions to the above mentioned issues. Apart from the use of the low solubility of a number of plasma proteins at low temperatures for purification purposes (e.g. cryoprecipitation of fibrinogen and factor VIII/von Willebrand factor complex, and other cold-induced pro-

tein precipitation techniques), low temperatures are of course of help in prevention of denaturation, enzymatic activation and proteolysis, as well as bacterial growth. So, cooling of whole blood and plasma, freezing of plasma and intermediate products, cooling to subzero temperatures (during the fractionation into immunoglobulins and albumin in the processing scheme according to Cohn [1], and freeze-drying (for optimal preservation of labile components during long-term storage) are widely accepted techniques since many decades now. As several of the latter techniques are dealt with by a number of experts in these symposium proceedings, the subject of this contribution will focus mainly on the primary processing steps of blood and plasma.

Procurement of whole blood

The preservation of plasma proteins during collection of blood and recovery of plasma is almost synonymous with the preservation of blood coagulation factor VIII. The high need for factor VIII, its lability and the limited supply of donor plasma all urge to optimally preserve its biological activity. The measures to achieve this may start already at the donor level. While it has been recognized for a long time that individuals with blood group A, B or AB have higher factor VIII concentrations in their blood compared to those with group O (100-125% versus about 85% of normal) [2], one could consider to apply a donor selection based on blood group. Although this seems a sensible approach for special applications (e.g. plasmapheresis), the need for factor VIII is such that donors with blood group O can not be omitted.

Somewhat more realistic for a regular blood transfusion system is physiologic stimulation of the factor VIII concentration of the donor's blood just before donation. Behaving as an acute phase reactant, factor VIII can be stimulated to levels 2- to 5-fold over normal by strenuous exercise [3] or by direct administration of hormones like vasopressin or its analogue DDAVP [4]. Treatment of mildly or moderately affected patients with hemophilia A or von Willebrand's disease with DDAVP has been quite successful [5], but stimulation of donors with this drug [6] has gained only limited application till now, as blood transfusion systems with voluntary, non-remunerated donors are reluctant in introducing this. The best example of donor stimulation by DDAVP is found in Sweden, where this has been introduced successfully since a few years. In a research study the higher level of factor VIII in the donated plasma as induced by DDAVP was shown to result in higher than normal recoveries in the final factor VIII concentrate produced from such plasma [7]. However, also in Sweden the use of DDAVP has been abandoned on ethical grounds now.

For a long time much effort has been spent in trying to preserve factor VIII optimally during collection of the blood. Two main issues play a significant role here: the type of anticoagulant and the way the blood is

drawn. It has been known for a long time that the use of oxalate or EDTA as anticoagulants was detrimental to factor VIII activity. We now know that this is due to factor VIII being a calcium-containing metalloprotein which loses its coagulant activity readily when brought into contact with calcium chelators of too high affinity, like EDTA [8]. Also citrate, generally used in anticoagulants like ACD and CPD, is exerting a denaturing action on factor VIII for the same reason. This has become apparent with the use of heparin as an anticoagulant, while leaving out citrate [9,10,11]. Although the increased basal activity level of factor VIII as reported by Rock et al [9,11] had been subject to some controversy in the literature [12,13], little doubt exists concerning the increased stability during storage of heparinized whole blood or plasma compared to their citrated counterparts [9,11,14,15]. The major role calcium ions play in maintaining factor VIII coagulant activity is probably most convincingly demonstrated by the partial renaturation of factor VIII that is effected by recalcification (and heparinisation) of blood or plasma when done within 4-6 hours after blood collection on a citrate anticoagulant [11, 14].

In spite of being an effective anticoagulant (in the absence of blood platelets), heparin has not been introduced on a large scale in plasma fractionation, as it is interfering with the production of plasma components other than factor VIII. However, Prowse et al [16] and Rock et al [17] have advocated lowering the citrate concentration to about half the original strength. This results also in higher factor VIII recoveries and stability, although lower than that seen with anticoagulation with heparin. Along similar lines Foster et al [18] maintain a certain level of free calcium ions (in a citrated system) to avoid unnecessary losses of factor VIII during the production of a factor VIII concentrate for clinical use.

The second point of concern is the way the blood is collected. Of course the venepuncture shall be flawless in order to limit activation of the coagulation system by tissue factor and/or contact with non-endothelial surfaces. Other causes of activation of coagulation have been attributed to inadequate mixing of the blood with the anticoagulant, to long donation times (too low blood flow) and to delayed stripping of the collection tube. Pflugshaupt and Kurt [19] showed that inadequate mixing with anticoagulant resulted in elevated concentrations of of fibrinopeptide A (fpA) in donated blood, fpA being one of the most sensitive markers of the generation and presence of thrombin in blood or plasma. Besides that they found that when such plasma was processed to an intermediate purity type of factor VIII concentrate, the factor VIII therein was less stable and the solubility was impaired. Also, small-pool cryoprecipitate prepared from plasma containing more than 30 ng/ml of fpA clotted more frequently.

A number of investigators since then have also made use of fpA measurements [20-24]. They confirmed the frequent presence of elevated fpA concentrations in blood donations, although Prowse et al [21] did not find a correlation with the way of mixing. However, they found

a correlation with long donation times (in excess of 10 minutes). This was confirmed by Huh et al [24], but it was disputed by Carlebjörk et al [20]. The latter group also did not find a correlation between high fpA levels and increased lability of factor VIII, but this may have been caused by the different manufacturing procedure of the factor VIII concentrate. Late stripping of the contents of the collection tubing into the blood bag was shown to be a possible cause of high fpA levels by two groups [21,23].

It is logical that activation of the coagulation system is to be restricted to a minimum by optimizing the way blood is drawn and collected. In spite of some conflicting results as discussed above, the test on fpA seems a valuable one in assessing this. Although the test is not suitable for mass screening puposes because of its laborious procedure, it can be considered to be a very sensitive means for validation of new blood collection procedures.

Procurement of plasma

When the blood is collected and adequately anticoagulated it should ideally be processed to cell concentrates and plasma without delay. This however, usually poses logistic problems. So, many studies have been dealing with the effects that cooling and storage for periods up to 24 hours have on the quality of the blood. Quite varying results have been reported with regard to factor VIII, when whole blood was stored at 4°C. There are two major reasons for this.

First, when in routine practice blood is cooled it makes a significant difference whether the entire bag is in direct contact with the cooling air or is somewhere in the middle of a pile of bags: the temperature history of the bags is seldomly known in detail. Second, when blood or plasma are stored at temperatures below 8-10°C spontaneous (cryo)precipitation of fibrinogen and factor VIII/von Willebrand factor complex starts taking place [25]. In combination with the uncontrolled drop in temperature mentioned above this leads to quite varying factor VIII concentrations in the plasma after centrifugation of the blood. However, losses of 30-40% are the average usually encountered when whole blood is stored for 16-24 hours prior to primary processing.

Finally, a third point of concern in storing whole blood is prevention of proteolysis which may be the result of (white) cell lysis below about 20°C. These considerations have led to a change five years ago in the treatment of whole blood collected by the mobile teams of our institute. Each blood collection is ended by transferring the needle from the vein into the rubber stopper of an evacuated sampling tube: all blood in the blood line is thus removed and replaced by anticoagulated blood from the blood bag. As soon as the blood has been taken from the donor the bag is placed on a precooled pack filled with 1,4-butanediol [26]. 1,4-Butanediol has its melting point at 20°C with a specific melting heat of 36 kcal/kg. This results not only in a more rapid and standardized

initial cooling but also in stabilisation of the actual storage temperature at 20°C. The bags are stored overnight in this way and are centrifuged the next morning. Quality control performed on bags stored in this way showed an average factor VIII concentration in 9 pools of 10 or 20 samples of 0.84 IU/ml (Table 1), while blood cooled on melting ice (final temperature 0-4°C) yielded only 0.45 IU/ml. In routine practice average factor VIII concentrations fall in the range of 0.74-0.77 IU/ml. Cell concentrates prepared from blood cooled and stored in this way fulfill all requirements for transfusion [26].

Another quality aspect of plasma is the extent to which it is contaminated with blood cells. The more cells present the higher the chance of release of proteolytic activity from these cells following freezing and thawing of the plasma. Also, a high contamination with cells may result in poor solubility of, for instance, small-pool cryoprecipitate, due to membrane fragments and denaturated nucleoproteins of the lysed white cells. The best quality plasma in this respect seems to be the plasmapheresis plasma obtained by membrane filtration, as this is essentially cell-free.

Primary processing of plasma

For proper long-term storage and to enable cryoprecipitation, plasma must be frozen. A number of investigators have addressed this issue. Generally speaking, plasma must be frozen at a relatively high freezing rate of about 2-4 cm/hour (about 30 minutes for a plasma bag) [27]. At low rates salt and protein gradients will form resulting in high concentrations in the middle of the bag (Figure 1). Cryoprecipitate prepared from plasma frozen at low rate will show a somewhat lower factor VIII recovery and, more significantly, an increased amount of other proteins, resulting in a low specific activity of factor VIII (Table 2) [27].

Storage of frozen plasma is another point of concern. Theoretically, when a solution is frozen and stored below its eutectic temperature little will happen to the frozen mass. Although no distinct eutectic temperature can be given for plasma, storage at or below –23°C to –30°C is considered to be safe. However, temperature insults, even for short periods

Table 1. Influence of cooling of whole blood on factor VIII in plasma.

Cooling temperature	Factor VIII (IU/ml)	Protein (g/l)	n
0–4°C	0.45 ± 0.06	63.7 ± 2.2	9 × 10
20°C	0.84 ± 0.10	65.6 ± 2.7	5 × 10 4 × 20

Data are given as mean ± SD.

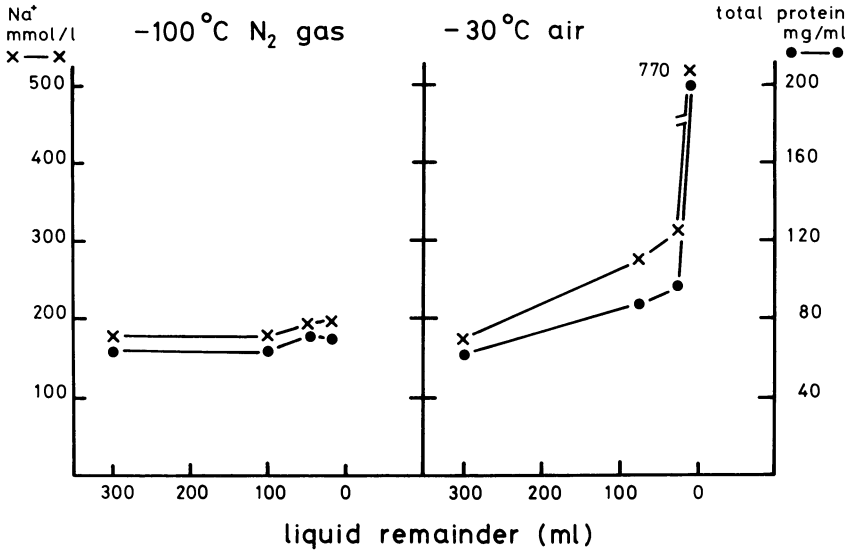

Figure 1. Salt and protein concentrations in the center of the liquid in a bag of plasma (300 ml) during freezing at two rates of freezing (about 12 minutes freezing time in N_2-gas and 3-4 hours in air of −30°C).

of time, should be avoided, as was convincingly demonstrated by Farrugia and Prowse [28]. Elevation of the storage temperature to −5°C, followed by a decrease to −40°C again shortly after that, resulted in increased amounts of protein in the cryoprecipitate made from such plasma.

Finally, thawing of plasma for preparation of cryoprecipitate is also critical. As reported before [27] as well as by many other groups [25, 29-33] quick thawing results in higher recoveries and specific activity of factor VIII compared to slow thawing methods. As was the case in the freezing of plasma, a slow rate of thawing results in formation of salt and protein gradients which presumably is the cause of factor VIII denatur-

Table 2. Influence of freezing rate of plasma on factor VIII and protein recovery in cryoprecipitate.

Freezing time* (hr)	F VIII recovery (IU/l)	Protein (g/l)	Specific activity F VIII (IU/mg)
20–30	433	34.9	0.21
4–6	490	29.5	0.29
0.33	513	23.7	0.38
0.12	458	22.0	0.38

* Time needed for complete solidification of 300 ml in a plasma bag.

[Data derived from ref. 27]

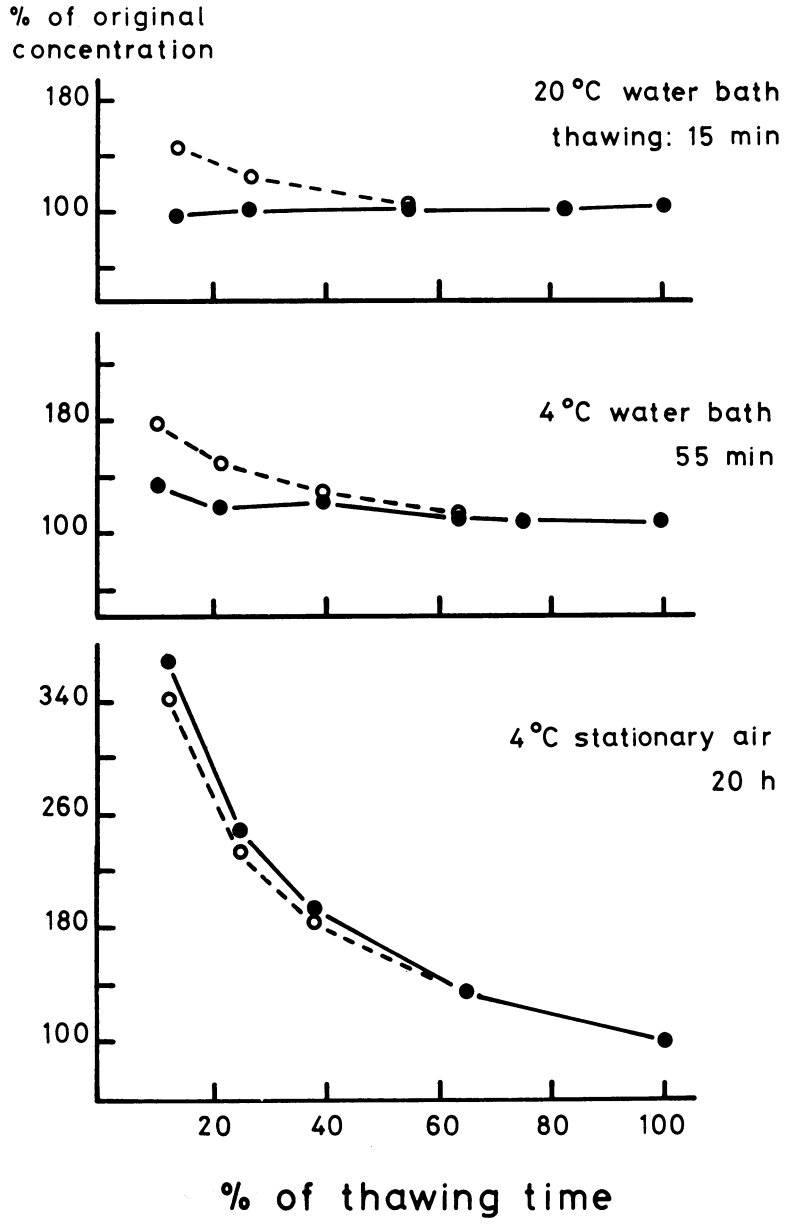

Figure 2. Salt and protein concentrations in the fluid phase of thawing plasma. Concentrations shown are the mean of those of Na^+, albumin, IgG, IgA, IgM and total protein. The broken line represents the data for plasma that had been frozen slowly (4-6 hours), the solid line for plasma frozen quickly (about 10 minutes). [Originally published in ref. 27]

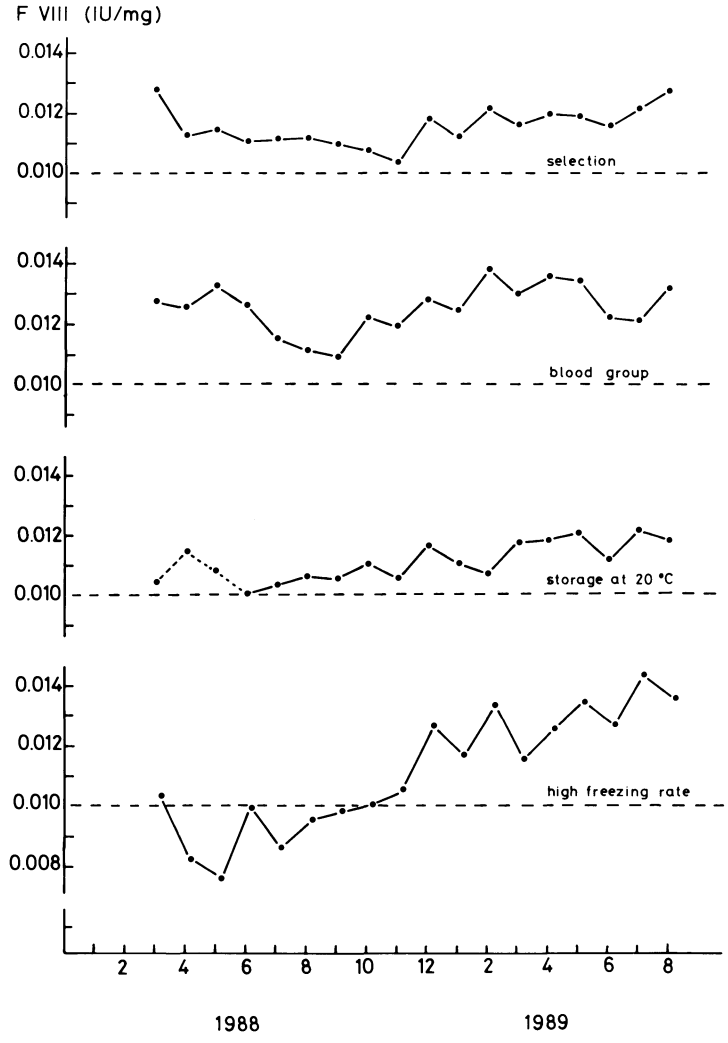

Figure 3. Specific activity of factor VIII in plasma from four blood banks as measured in a sample taken from the centre of 20 frozen plasma bags. See text for details.

ation and precipitation of other, unwanted proteins (Figure 2) [27]. So, for optimal primary processing of plasma a high rate of both freezing and thawing is a prerequisite: a high freezing rate can not compensate for slow thawing, and, vice versa, rapid thawing can not compensate for slow freezing.

The importance of a few of the items discussed above is demonstrated by the results of our quality control program of the fresh-frozen plasma

that regional blood banks ship to our institute for fractionation. This quality control is done on every shipment by cutting a sample of plasma-ice from the center of 20 plasma bags. After complete thawing and pooling of the samples both factor VIII concentration and total protein content are measured. The quotient of the results (specific activity of factor VIII) is an indicator for the quality of the plasma. A specific activity of 0,010 IU/mg is arbitrarily set as the limit that distinguishes good from poor quality plasma.

Figure 3 shows the results that were obtained for four blood banks from March 1988 till August of 1989. The first one, during a period in 1988, selected the best quality plasma for their in-house production of cryoprecipitate leaving plasma of lower quality for fractionation (though still meeting the requirement of 0,010 IU/mg). The second one faced a higher demand for blood of group O during the summer-time, resulting in a temporary fall in the average factor VIII concentration. The third one adopted the procedure of cooling donated blood rapidly to 20°C by using precooled units with 1,4-butanediol and storage (overnight) at that temperature. This resulted in a gradual increase in specific activity since then. The fourth one finally is a good example of the improvement in plasma quality when a slow freezing process is replaced by a quick one (in this case freezing in ethanol at –40°C).

Conclusion

For saving the valuable therapeutic components present in human blood plasma many precautions have to be taken. In the past many parameters have been subjected to extensive investigation. Although the results have not always been unequivocal, it has now become clear to a great deal how to optimally handle blood and plasma (as well as donors).

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RESIDUAL WATER CONTENT IN A LYOPHILIZED, HEATED COAGULATION FACTOR CONCENTRATE

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Introduction

Control of residual water content (rwc) is an important feature of any lyophilization process and becomes critical when dried products are subsequently severely heated at 80°C for 72 hours in order to inactivate viruses. Measurement of rwc by traditional methods [1-3] is time-consuming and destructive, limiting the numbers of measurements which can be made and preventing alternative analyses on vials for which rwc has been determined. We have investigated the use of a non-destructive system for rwc determination based on near infrared absorption. We have used this method as well as Karl Fischer determinations to investigate several aspects of rwc in our heated factor VIII concentrate, 8Y. We have identified some parameters in addition to the standard plant-related functions (e.g. product and condenser temperatures) which contribute to rwc in the dried concentrate. We have looked at the effects of variation in rwc upon the biological activity and solubility of the product after heat treatment. We have also used the infrared method to survey entire batches of product, which has enabled us to look at within- and between-batch variation in moisture content.

Materials and methods

Materials

Factor VIII concentrate was prepared by our standard process [4]. The formulation buffer was 0.1 M sodium chloride, 0.01 M sodium citrate, 0.01 M Tris, 1.2 mM calcium chloride, 1.5% (w/w) sucrose, pH 6.9. Variations in standard formulation are specified in relevant results sections.

Vials used for lyophilization were 50 ml DIN, type 1 (SGD, Mers-les-Bains, France). Our standard stoppers were Pharmagummi chlorobutyl PH701-40 (West Pharmarubber, St. Austell, UK). In one experiment

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three alternative stoppers were included: Selgas bromobutyl 544-04 (Wentworth Medical Supplies, Horsham, UK); bromobutyl FM157-1 (Helvoet Pharma, Alken, Belgium); chlorobutyl 544 FU (Schubert Systems, Havant, UK). Stoppers were washed, autoclaved and heated at 80°C for 16 hours before use.

Plant

Three types of freeze-dryer have been used, all capable of similar control. Large-scale batches were dried on Lyomax 2-SS or EK8 dryers and small-scale experiments were performed using a Lyoflex 06 (all from Edwards High Vacuum, Crawley, UK).

Lyophilization

After sterile filtration and dispensing in 10 g aliquots, vials were loaded on to the dryer shelves at 20°C. Shelves were then cooled to –50°C over 80 min and the product held <–50°C for 10 h. After evacuating the chamber (0.25 mB), primary drying was initiated by warming the shelves to –10°C. Shelves were held at –10°C for 20 hours and were then warmed to 30°C over 15 hours. The product was held at 30°C for a further 20 hours, with a reduction in the vacuum to 0.06 mB for the final 10 hours. Vials were then stoppered under vacuum, removed from the dryer and oversealed. Any variations in this standard method are specified in the relevant results sections.

Heat treatment

Vials were heated in ovens at 80°C for 72 hours.

Determination of rwc

Two methods have been used:

Using the Karl Fischer method [1], 20 ml dry methanol was introduced into the weighed vial by syringe. After sonication and overnight extraction, water was quantitated coulometrically and the weight of the lyophilized plug determined by difference. Thus the method assays mg water/vial and allows calculation of % rwc also.

Rwc has also been measured by near infrared absorption using a Quadra-Beam analyser (Moisture Systems Corp., Hopkinton, MA, USA). In this instrument a rotating filter wheel allows alternate passage of infrared (ir) beams of three wavelengths into the product in its sealed vial and reflection to a detector. By using one wavelength that is specifically absorbed by water and two other adjacent wavelengths that are not absorbed either by water or by the product, the amount of light absorbed by the water in the product can be determined. Calibration of this instru-

ment was carried out by preparing duplicate samples of similar water content (as measured on the *un*standardized Quadra-Beam) at each of several rwc. After determination or rwc by the Karl Fischer method on one of each pair, maximum and minimum values were set on the Quadra-Beam using the second vial of the highest and lowest rwc. The remaining vials of intermediate rwc were then analysed to ascertain whether there was a correlation. There is no determination of plug weight in this non-destructive method, and therefore residual water was calibrated against the Karl Fischer measurement of total mg water/vial, and is quoted as mg/vial.

All values for rwc quoted in this communication refer to mg water/vial as determined by the Karl Fischer method. Where the measurement has been made by Quadra-Beam, values have been converted using the calibration correlation. For the 8Y concentrate, 2.8 mg water/vial corresponds to 1.0% (g/g) rwc.

Miscellaneous

Factor VIII activity was measured with a two-stage clotting assay [5] using the British Working Standard (87/682). Activity loss after heat treatment was always determined by parallel assay of samples from heated and unheated vials.

Total protein was measured using the Biuret method [6].

Results

Calibration of the Quadra-Beam moisture analyser for 8Y

There was a linear relationship between Quadra-Beam and Karl Fischer determinations over a wide range of rwc (Figure 1). The vials included in this calibration exercise had a range of total protein concentrations (2.7-8.0 mg/ml).

Within- and between-batch variation of rwc

A survey of rwc in every vial from a batch of 8Y showed an approximately normal distribution (Figure 2; mean = 2.45 mg, SD = 0.30, n = 547). Other batches dried on the same plant were also surveyed and each of these also showed an approximately normal distribution. Comparison of four batches (Figure 3) illustrates some differences between batches in both mean rwc and in the width of the normal distribution. In this small survey there was no obvious relationship between mean and standard deviation.

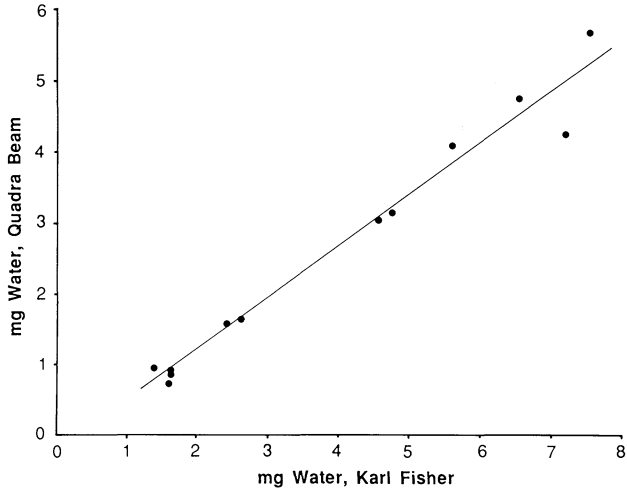


Figure 1. Calibration of the Quadra-Beam moisture analyser. Each point represents a vial of 8Y for which rwc was first determined by infrared absorption and then by the Karl Fischer method.

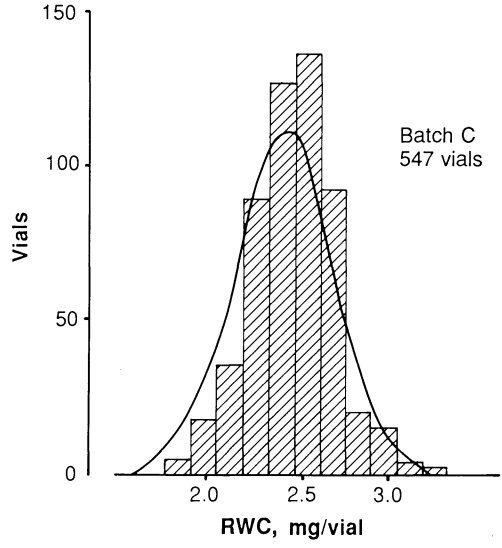


Figure 2. Distribution of rwc in a single batch of 8Y.

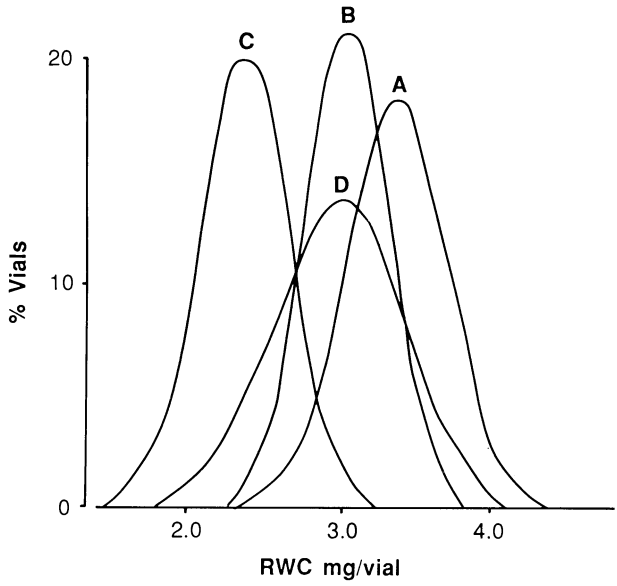
Factors which determine rwc

Using our standard freeze-drying programme, with fixed final product and condenser temperatures, we have found that rwc will also depend upon at least three other parameters.

Stoppers are sterilized before use by autoclaving, during which procedure they absorb a significant amount of water which is not removed by drying at 30°C and which is subsequently released into the vial during the terminal 80°C heat treatment. An investigation of water removal from four different stoppers showed that removal approached completion only after 24-48 h at 80°C (Figure 4). Bromobutyl formulations absorbed less water than chlorobutyl ones and released the absorbed water more rapidly.

The rate at which the product was cooled to –50°C after freezing and before lyophilization had a pronounced effect on rwc (Table 1).

A survey of 90 batches of 8Y illustrated a clear inverse relationship between total protein concentration and residual water (Figure 5).



Batch	n	Mean	SD	Skewness
A	153	3.37	0.32	-0.455
B	349	3.06	0.27	-0.023
C	547	2.45	0.30	2.98
D	845	3.00	0.43	0.202

Figure 3. Comparison of best fit normal distributions of four batches of 8Y.

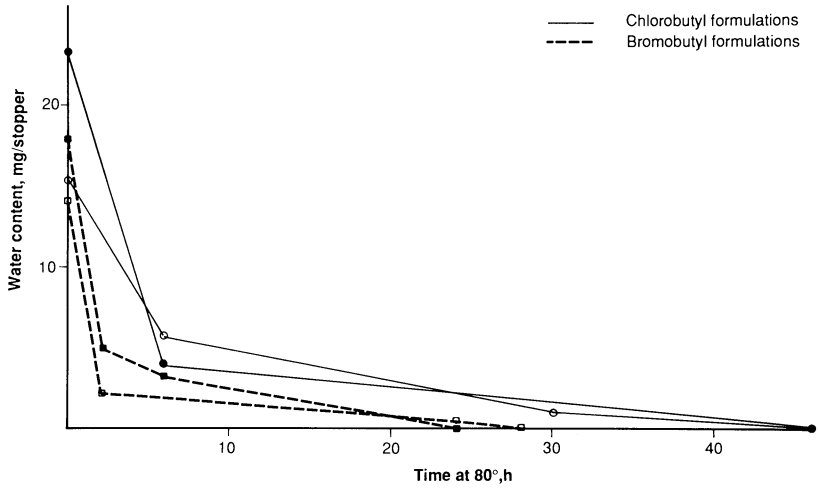


Figure 4. Water removal from vial closures. 50 Closures were washed, autoclaved and dried at 30°C. After weighing, they were dried at 80°C and weighed at timed intervals. Four different closures were used: PH 701-40 (○); 544-04 (■); FM 157-1 (❑), and 544 FU (●).

The rwc increased when the concentration of sucrose in the 8Y formulation was raised (Table 1).

Effects of rwc on the heat-treated product

In the range 2-8 mg water/vial, there was no correlation of solubility of factor VIII recovery with rwc for *un*heated samples. However, heating at 80°C for 72 h caused changes in solubility and loss of factor VIII activity, both of which were affected by the level of residual water present. Up to 8 mg water/vial, the solubility of the heated product was not adversely affected by rising rwc; the product was completely soluble, and there was

Table 1. Effects of cooling rate and sucrose concentration on residual water content of 8Y.

Time to –40°C, h	% (w/w) sucrose	rwc, mg/vial
1	1.0	1.3
3	1.0	1.3
9	1.0	1.9
18	1.0	2.9
1	1.5	4.3
3	1.5	5.3
9	1.5	5.6
18	1.5	5.5

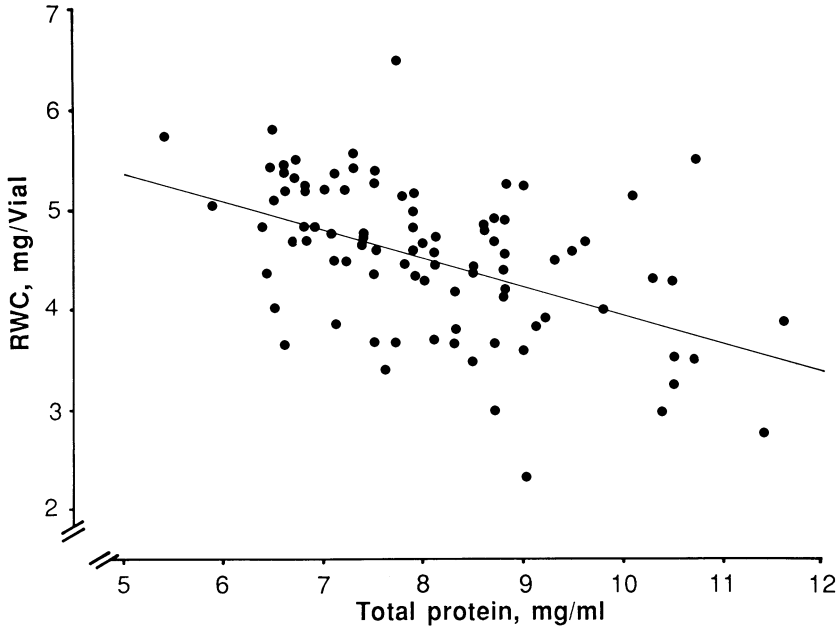


Figure 5. Variation of residual water content with total protein concentration. Data are from 90 batches of 8Y. In each case rwc was determined on a single vial by the Karl Fischer method.

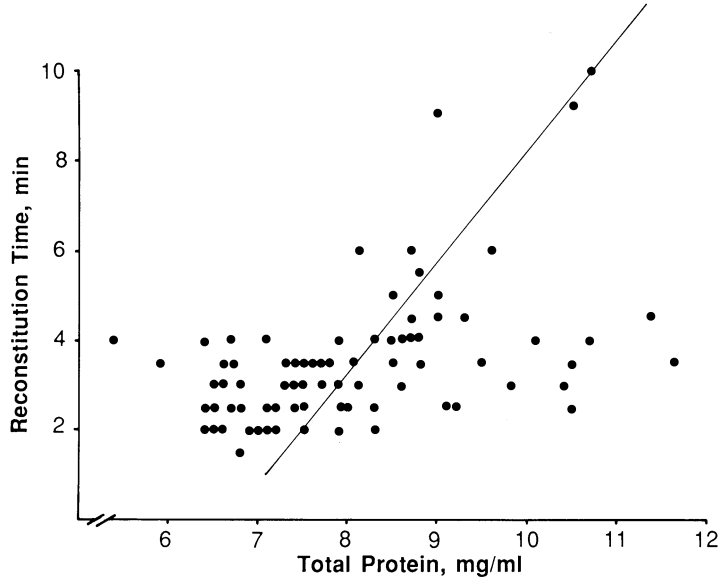


Figure 6. Variation of reconstitution time with total protein concentration. Reconstitution time was the time taken for complete resolution at 20°C. Data are from 90 batches of 8Y.

a weak coorelation between the time taken for reconstitution and the protein concentration (Figure 6). However, above about 8 mg water/vial, solubility was incomplete and turbidity was substantially increased.

The correlation between rwc and loss of activity suffered after heat treatment was also weak but showed an extra 2% loss of factor VIII activity for each additional mg of water (Figure 7).

Discussion

The most interesting aspect of this work has been the use of a non-invasive method of residual water determination. Results show convincingly that water detected by the Quadra-Beam near infrared analyser is directly comparable to that extracted in Karl Fischer determinations. Moreover, the sensitivity of both methods is similar. The major difficulty in using the infrared method is the initial calibration with its requirement for vials of known rwc at the extremes of the range of interest. Measurements of the water content of two other products using the 8Y-calibrated Quadra-Beam have shown different linear correlations with Karl Fischer determinations for these products and indicate that each product will need its own calibration to quantify rwc [L. Winkelman, unpublished observations]. It may also turn out that different reference wavelengths are required for various formulations. The data for 8Y show that varying protein concentration in the 8Y formulation did

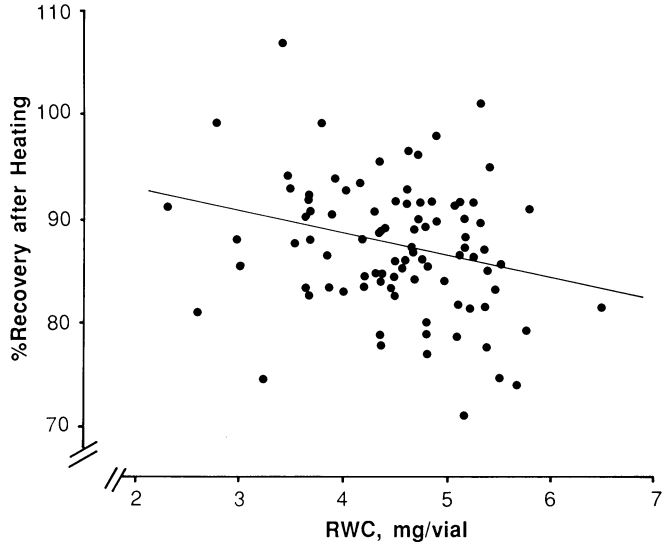


Figure 7. Variation of recovery of factor VIII activity after heat treatment with residual water content. Dried vials were heated for 72 h at 80°C and the activity of heated samples compared to that of unheated controls from the same batch. Data are from 90 batches of 8Y. Rwc was determined on a single vial from each batch by the Karl Fischer method.

not affect the calibration; however, we have not yet explored the effects of other changes or altered freeze-drying conditions on the calibration.

Use of the infrared method has allowed a complete survey of rwc in large batches of factor VIII. The fact that the distribution of rwc across a dryer load of several hundred vials approached a normal distribution gives confidence in both the freeze-drying plant and the operational programme. The ability to analyse every vial will provide a useful tool in the development and/or modification of lyophlization programmes in future.

The ability to measure rwc non-destructively in vials which can then be assessed in other ways will also be valuable. We are currently using the method in spiking experiments being carried out to quantify virus inactivation during heat treatment; spiked vials cannot be dried in production plant and comparability must be illustrated.

The results of the investigation of parameters which affect rwc allow us to have better control over the lyophilization process. Drying the stoppers at 80°C before use is obviously important if terminal heat treatment is intended. Knowledge of the effects of the cooling rate after freezing has led us deliberately to programme the rate of shelf cooling so that small batches will not reach –50°C more rapidly than large ones. The effects of protein concentration on rwc confirm earlier pilot work [7] and highlight the importance of controlling this parameter, often a variable one in intermediate purity factor VIII concentrates.

We have found a threshold level of residual water above which critical solubility problems can be expected and have established that, below that threshold of about 8 mg water/vial, further reduction of rwc and loss of activity after heating also confirmed earlier results [7]. The fact that the correlation was only a weak one suggests that there are other influences which also affect recovery of activity.

We are currently using the techniques described here in an investigation of the effects of variation in rwc on virus inactivation in the 8Y concentrate. We hope to extend the use of infrared rwc measurement to other products in the near future.

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2. May JC, Grim E, Wheeler RM, West J. Determination of residual moisture in freeze-dried viral vaccines: Karl Fischer gravimetric and thermogravimetric methodologies. J biol Stand 1982;10:249-59.
3. Jewell JE, Workman R, Zeleznik LD. Moisture analysis of lyophylized allergenic extracts. Devel biol Stanard 1977;36:181-9.
4. Winkelman L, Owen NE, Evans DR, et al. Severaly heated therapeutic factor VIII concentrate of high specific activity. Vox Sang 1989;57:97-103.
5. Biggs R, Eveling J, Richards G. The assay of antihaemophilic globulin activity. Br J Haemat 1955;1:20-34.

6. Gornall AG, Baradwill CJ, David MM. Determination of serum protein by means of the Biuret reaction. J biol Chem 1949;177:751-66.
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DISCUSSION

S. Holme, P.J.M. Salemink

J.C. Bakker: Dr. van Assendelft, I found your study very interesting and of very practical value. But I did not quite pick up which parameters did you test. You told about the recovery, so I suppose electronic counting and trypan-blue exclusion. But did you also look at more sophisticated function tests, for example a function that might be affected: IgG production of B lymphocytes.

O.W. van Assendelft (Atlanta, GA, USA): That would, of course, have been a possibility, but we did not, because we use the lymphocytes that we isolate primarily for surface marker studies and for the isolation of HIV.

C.F. Högman: Dr. Das, platelets are particularly sensitive to osmotic stress and I wonder whether you or anybody has an experience using for instance a dialyser to introduce DMSO and also remove it in order to make this procedure smoother and less damaging to the cells.

P.C. Das (Groningen, NL): The hypotonic shock response is a pretty good test for fresh platelets. For example one could utilize the hypotonic shock response and follow the response drop in liquid stored platelets. But the moment you add DMSO being a penetrating agent, there remains some of it after freezing and thawing. In my opinion this test does not reflect the quantitative functional reality of the frozen platelets. Certainly, in our hands the *in vivo* recovery does not correlate well with the hypotonic shock response.

H.T. Meryman: We have had a great deal of experience with looking at the osmotic tolerance of platelets. Unique among blood cells, they have essentially no tolerance to changes in osmotic pressure. We find that as we reduce or increase the osmolarity, all of the functional tests start to fall off and at an osmolarity of 600 milliosmoles, even briefly for a few seconds, the function tests are roughly half of the control. Dialysis is a much better way of introducing either glycerol or dimethylsulfoxide. When these are introduced by dialysis, over a period of about 10 minutes

for DMSO or half an hour for glycerol, one can double the recovery of platelets following freezing and thawing. The problem of course is that, from a technical standpoint, as a routine procedure this will require a very substantial reconstruction of the process, the development of new equipment, new bags. We have not as yet sensed that there is a sufficient demand for this product to warrant investing that kind of resources, but it is a very useful approach to the introduction and removal of a cryoprotectant.

F. Beaujean (Creteil, F): Dr. Das, what is your dilution of DMSO. How do you add DMSO to preserve platelet suspension.

P.C. Das: We use 10% DMSO in the plasma. When added slowly, the final concentration is 5%.

W.J. Armitage: I would like to agree with what dr. Meryman has just said about the osmotic tolerance of platelets. They are indeed highly susceptible to osmotic stress, but their sensitivity is very dependent both on temperature and on the nature of the solute used to induce shrinkage. For example, platelets are more tolerant of osmotic stress at 0°C than at 25°C or 37°C, and at 37°C they are much more tolerant of shrinkage induced by the nonelectrolyte sucrose than of shrinkage induced by sodium chloride.[1] But, when dealing with the influence of osmotic stress during the addition and removal of glycerol, one also has to take into account the change in the permeability of the platelets to glycerol. Although platelets are much more tolerant of osmotic stress at 0°C, they are, unfortunately, virtually impermeable to glycerol at this temperature.[2] So, there are many factors to take into account when dealing with osmotic stress during the addition and removal of cryoprotectants.

J.N. Kearney (Wakefield, UK): Dr. van Assendelft, you showed us a graph of cooling rates of vials that were insulated compared with unprotected vials. Between the temperatures of –10°C and –40°C, those curves where remarkably similar. Have you looked at the viability of lymphocytes cooled without any sort of insulation?

O.W. van Assendelft: That graph showed you the two typical temperature recordings for vials placed in a book bag. It had a fairly straight line which indicated a controlled rate at 1°C per minute. On the extreme left hand side there was, shall we call it, an aborted tracing which was from an unprotected vial and in which the temperature plunged rather violently down.

1) Armitage WJ, Parmar N, Hunt CJ. H Cell Physiol 1985;123:241-8.
2) Armitage WJ. J Cell Physiol 1986;128:121-6.

J.N. Kearney: Unless I was completely mistaken, the book bag cooling curves averaged 1°C per minute, but between the temperatures of –10°C and –40°C, the cooling rate was somewhere between 1.5°C and 2°C per minute rather similar to the unprotected vials. This is precisely the part of the cooling curve which is critical for subsequent viability of cells.

O.W. van Assendelft: If you look on that graph carefully, the tracing in the unprotected vial went down much faster.

B. Kubanek (Ulm, FRG): Dr. Beaujean, you showed very nicely that you can standardize freezing of stem cells, but you also showed that you cannot standardize the CFU-C assay. What do you rely on in daily practice: The count of mononuclear cells or the CFU-C assay?

F. Beaujean: Routinely we control the freezing process by the mononuclear cell recovery. The CFU-GM assay is used systematically, but not routinely. So not in every procedure.

B. Kubanek: But what would you rely on? If you had a great variation in your CFU-C assay, as we all have, and you have a marrow with 30% recovery by the CFU-C assay, but a normal mononuclear cell count, would you rely on the assay or on the mononuclear cell count?

F. Beaujean: That is difficult to answer. In the majority of the situations the CFU-GM assay is used to predict engraftment *in vivo*, but in some situations especially after *ex vivo* treatment by chemotherapy, where we have total depletion of CFU-GM we use recovery of mononuclear cells to determine the recovery from freezing.

D.E. Pegg: Could I come back to the question of the osmotic fragility of platelets and cryopreservatives. I do not wish to disagree with Dr. Meryman or Dr. Armitage, platelets undoubtedly are extremely sensitive to osmotic shock. But the message cannot possibly be that they are totally disrupted by any sort of osmotic shock, otherwise you could not cryopreserve them with DMSO. There is in the literature some information from Dr. Armitage[1-3] and from François Arnaud[4] from Cambridge on the permeability characteristics of platelets for glycerol, propyleneglycol and water, from which data it is possible to calculate what volume changes are produced under particular schedules. Therefore, if you

1) Armitage WJ, Parmar H, Hunt CJ. J Cell Physiol 1985;123:241-8.
2) Armitage W.J. J Physiol 1986;374:375-85.
3) Armitage WJ. J Cell Physiol 1986;128:121-6.
4) Arnaud FG, Pegg DE. Cryobiology 1990;27:107-18.

have data on the osmotic tolerance of platelets and you have limits beyond which you are not prepared to allow the volume either to expand or to shrink, then you can design techniques which avoid the practical point of view. Even glycerol can be got in and out of platelets. We now have developed a technique with 1.4 molar glycerol and *in vitro* tests are giving as good results as you get with the DMSO technique.[1] We do not yet know how good they are going to be clinically, which of course is the proof of the pudding.

C.Th. Smit Sibinga: Dr. Beaujean, the remark you made on the storage temperature of cryopreserved stem cells; in the literature it is mentioned that that should be below –150°C. Could you tell me what is the actual effect of storage over a given period of time on stem cells, the way you process them as compared to –150°C and lower. So, what actually is the effect on the colony forming capacity of stem cells when stored at for instance a –120°C or maybe –90°C instead of below –150°C?

F. Beaujean: I think that more details are given about longer preservation of CFU-C when the frozen bone marrow is kept at a temperature below –150°C. In our experience we have kept bone marrow frozen for 8 years in liquid nitrogen. Recently we used this bone marrow with good CFU-GM recovery, but culture conditions are quite different between these two periods and it is difficult to compare to have a real idea about the recovery.

C.Th. Smit Sibinga: For the time being we will remain with the recommendation, but maybe there is somebody else in the audience who could give some further comment, because this is still a puzzling point.

F. Beaujean: Dr. Stiff[2] kept frozen marrows in –80°C freezer with good results in terms of storage; the time of storage was about two years.

C.Th. Smit Sibinga: Dr. van Assendelft, you know that lymphocytes have become more and more of interest as a commodity for enhancing the immune therapy in certain cancers, so using them as kind of commando cell to be stimulated and activated by cytokines and given back to the patient. Do you have any information based on your experiments whether the lymphocytes and mononucleated cells you preserve, still have the potential to be activated by lymphokines or whether that potential is lost following your procedure.

1) Arnaud FG, Pegg DE. Thromb Res 1989;53:585-94.
2) Stiff PJ, Koester AR, Weidner MK, Dvorak K, Fisher RI. Blood 1987;70:974-8.

O.W. van Assendelft: Whether or not they can be activated by lymphokines I do not know. However, I do know that they can be stimulated by various mitogens.

F. Beaujean: I just would like to make a comment. Recently I think a Japanese team[1] studied the capacity of frozen lymphocytes to be stimulated by Interleukin-2 after freezing and thawing. They seem to have very good results for the stimulation after freezing.

C.Th. Smit Sibinga: It might be of importance in the near future to collect the lymphocytes in a slightly earlier stage and the patient being in a better condition rather than waiting till the cancer is in the end stage and the patient in a poor condition.

F. Beaujean: Of course, I think it is preferable to store lymphocytes before stimulation.

R. Mitchell: You can certainly get the lymphocytes to respond. We have been taking lymphocytes from peripheral blood mainly in people who have been boosted to produce monoclonal antibodies. If you recover the lymphocytes from these people by lymphocytapheresis, there is undoubtedly evidence that these cells are certainly dividing, as you can make very good clones from them.

H.T. Meryman: Mrs. Winkelman, I want to congratulate you on solving a problem that has plagued freeze-drying for decades: To develop a quick and reproducable method for measuring residual moisture is immensely valuable. Does any portion of the distribution in residual moistures in your curves represent a distribution in the quantity of material in the vial?

L. Winkelman (Elstree, UK): I think not. We filled at very tight weight parameters, something like 10 grams plus or minus 0.1. So I think this will not effect the amount of residual water. When you rotate the vial in the quadrabeam, you do get small variations. There may be a difference in the surface of the plug on the bottom and there may be variation in the glass thickness, which will give you slightly different readings and these probably contribute to the spread that you see.

P.J.M. Salemink: May I comment on this point also. We have been talking on variations in residual moisture from batch to batch as well as variations in residual moisture from one vial to another within the same

1) Kawai H, Komiyama A, Katho M, Yabuhara A, Miyagawa Y, Akabane T. Transfusion 1988;28:531-5.

batch. But in addition, we also have to be aware of variation in residual moisture within a single vial, because there can be heterogeneity over the vertical line in a single vial. This is a point that is not very much given attention sofar.

C.F. Högman: Dr. Over, first about the apparent discrepancy about FpA. I was also struck by that when I read these papers, but I do think that there might be an explanation in the time factor, because it may depend on when you have taken the samples. Prowse and collaborators[1] took their samples fairly quickly after blood collection. It might well be that if you store the blood for some time, thrombin and FpA are generated. If you look at plasma which is stored at 4°C, for instance like Carlebjörk et al[2] did some years ago, you may find that sometimes there is both FpA and kallikrein development in the plasma. This may come as late as after two weeks and then develop further. We have thought of the possibility that something has happened already during blood collection, but because of the low Ca^{++} concentration it may take quite a long time before you actually see the effects. We recently looked at a cryoprecipitate depleted plasma, which has of course been frozen and thawed. It was found that factor VII was quite often activated. So, after about one or two weeks, there was quite a decrease probably due to cold activation. Also sometimes there was kallikrein formation and it was striking that some of the plasma really behaved rather individually. We have no clear explanation why there was quite a strong activation in some of the plasma's.

Finally, I come to the plasma in whole blood. Some may argue that you should use whole blood because patients sometimes need plasma also. But I think it should be pointed out that the plasma in whole blood, if the whole blood has been stored for some time, is very poor because there is decrease of different factors, but also deterioration of leukocytes. So, for instance you can find histamine and you could probably find a lot of other leukocyte constituents in that plasma. This is I think a good argument to produce components and then store the components under relatively optimal conditions.

J. Over: Just to avoid misunderstanding; when I spoke about storage of whole blood I meant for a maximum period of 24 hours, because this is just done for logistical reasons. Blood that is collected in the evening can be stored overnight without damage either to cells or to factor VIII. You are talking about longer time periods in storing whole blood.

C.F. Högman: Yes of course, I meant when whole blood is being stored for let us say one or more weeks.

1) Prowse CV, Bessos H, Farrugia A, et al. Vox Sang 1984;46:55-7.
2) Carlebjörk G, Blombäck M, Åkerblom O. Vox Sang 1983;45:233-42.

J. Over: Two other remarks if I may. First the FpA. The message I got from those literature data is that there is no generation of FpA at least not directly after donation until, as you told, two weeks storage. Then you can see an increase in FpA levels. However, I was referring to data concerning only a short period of time after collecting the blood.

The second remark relates to the kallikrein generation. I think this may be due to partial inactivation of C_1-esterase inhibitor. This is less active in the cold and may then be less active in inhibiting kallikrein, which is then activating certain factors and so on.

R. Mitchell: Dr. Over, traditionally we have all been collecting plasma and processing on the same day, usually within 6-8 hours. What you are describing is quite revolutionary, because if we process the next day then, of course, we would save considerable amounts of manpower overnight. Was that one of the reasons that you chose to do this method in Amsterdam?

My other question is if you follow that system, how do you make your platelets and how do you make your cryo? I always thought that blood is a culture plate waiting to be colonized, and you are storing it overnight at 20°C. In your paper it is said n=30,000 when doing bacteriology. I do not know if you actually monitored 30,000 donations or if this was an extrapolation from a much smaller series that you reported in the preamble to the paper. I really just want to know the enormity of what you are saying. You have done many thousands now and you told us that because of some scientific evidence, you could keep plasma to the following day. When you told your technicians that, who normally work with you at night, what did they say. Were they delighted or were they saying, but I am going to be made redundant, I have got no job.

J. Over: I thank I can only speak for our own collection system which is focussed on plasma and not on cells. Our teams are going out in the evening to collect blood and transport it back the same evening. Up till 1984 we stored that blood at 10°C, because we were aware of the fact that below 10°C a precipitate would be formed with the result of losing factor VIII. The reason to change to 20°C instead of 10°C was just the consideration that we thought that the platelets and leukocytes were at least better preserved at that temperature during a short time period, and that perhaps the factor VIII yield could be improved. It did not make a change to the staff, because they are still doing the same thing: Processing the blood the next morning. The only difference is the temperature, at which the blood has been stored.

R. Mitchell: The other question I wanted to know was the bacteriology of all of this. Of the 30,000 bags stored at 20°C how many of them have been examined and what were the results. Were they examined aerobically and anaerobically, were they examined at a series of temperatures from 4°C to 37°C and what were the results? It is very important.

J. Over: I agree. A number of 800 platelet concentrates prepared from blood stored in this way has been analysed for bacterial contamination.[1] One was found to be positive for *Staphylococcus epidermidis*, possibly introduced by improper sampling. For small-pool cryoprecipitate prepared from this blood (all bottles tested in-process for unsterility) no rise in the frequency of unsterility has been found (annual production about 50,000 for 5 years now).

P.C. Das: Mrs. Winkelman, it is an excellent innovation that you presented with regard to the water content. But can you tell us how easy is it to adopt this to a simple freeze-drying apparatus and what does it cost?

L. Winkelman: You do not need to gear it to a freeze-drying apparatus. You simply put your vial into the beam as outlined. You have to make an adaptor for whatever size of vial you have. You need a fairly large plug surface to get sensitivity. The quadrabeam needs to see enough water molecules to register. We have a 10 ml plug which has a couple of centimetres diameter. You can get slightly smaller than that. The smaller you get, the less sensitivity you have. The instrument costs about £ 7000,–.

P.J.M. Salemink: Can you comment on the detection limit of this measuring method. What is the lowest detection limit, approximately.

L. Winkelman: Below 1 mg of water in that vial.

C.Th. Smit Sibinga: Mrs. Winkelman, I was looking at the description of your procedure in loading the freeze-dryer. You load onto the shelf at 20°C, so the shelf temperature at that point in time was 20°C. Is that correct?

L. Winkelman: That is correct.

C.Th. Smit Sibinga: What was the temperature of the material you loaded onto the shelf.

L. Winkelman: That was also 20°C.

C.Th. Smit Sibinga: Then you cool it down to –50°C in 1.3 hours?

L. Winkelman: That is correct.

1) Pietersz RNI, de Korte D, Reesink HW, Dekker WJA, van den Ende A, Loos JA. Vox Sang 1989;56:145-50.

C.Th. Smit Sibinga: Following Dr. Over's presentation would not we call this slow-freezing and what would be the impact then on the homogeneity of the frozen mass of 1 cm in your vials.

L. Winkelman: Well, the freezing is in fact quite rapid, it may be slow getting down to –40°C, but the phase changes quite rapidly. It supercools 5-10°C and then freezes rapidly and looks homogeneous. I cannot say more than that.

C.Th. Smit Sibinga: But actually what did you find in terms of homo- or less heterogeneity of the cake relative to the water content? You measured a certain amount in mg per vial; the point which was made yesterday by Dr. McIntosh from Edinburgh, who showed the three vials with one homogeneous and the other two far less homogeneous in cake substance. What actually was your observation?

L. Winkelman: The measurement gives us no indication of heterogeneity within a vial.

C.Th. Smit Sibinga: If I may ask another question regarding the platelet freezing. Dr. Pegg mentioned that propylene glycol (PG) at low concentrations is a superb protectant for freezing platelets. Could he probably say a little more about that and have there any function and clinical studies in the meantime come up with this cryoprotectant as opposed to DMSO.

D.E. Pegg: Propylene glycol is a terrible cryoprotectant for platelets. We found that it apparently had quite a reasonable toxicity on exposure and removal at temperatures above zero.[1] But when we tried to use it for cryopreservation, we found that the results were appaling. We had the anomalous result that we got the best recovery with the lowest concentration of propylene glycol and we concluded that the problem lay in a very steep increase in toxicity of this compound for platelets as it was concentrated as ice formed during the freezing process.[2] We also have evidence which is consistent with that from rabbit kidneys.[3] That may very well not be true for all systems, as Dr. Armitage[4] has got some good results with corneal endothelium using propylene glycol, and of course it is used in human embryo preservation. So, it probably is not a general property, but certainly for platelets, in our hands, it was terrible.

1) Arnaud FG, Hunt CJ, Pegg DE. Cryobiology 1990;27:119-29.
2) Arnaud PG, Pegg DE. Cryobiology 1990;27:130-6.
3) Jacobsen IA, Pegg DE, Starklint H, Hunt CH, Barfort P, Diaper MP. Cryobiology 1988;25:285-99.
4) Rich SJ, Armitage WJ. Cryobiology 1990;27:42-54.

P.J.M. Salemink: You are talking on toxicity Dr. Pegg, could you define the criteria of toxicity which you use in your laboratory.

D.E. Pegg: I am using the term loosely, that is correct. I am referring to the effect of propylene glycol upon the particular tests that we used. So, in the case of the platelets these were *in vitro* tests, ADP-induced aggregation, hypotonic stress test and the morphological appearances by electronmicroscopy. In the case of the rabbit kidney it was a totally different set of function tests of course; ultimately, the ability of the kidney to support an animal which had its other kidney removed. I used the term loosely, and perhaps inappropiately to describe any deleterious effect, whether it is a strictly chemical toxic effect or whether it is actually an osmotically-induced phenomenon.

W.J. Armitage: I would like to return to the question of the correlation between *in vitro* tests of platelet activity and *in vivo* function tests following cryopreservation. If I could ask Dr. Das and Dr. Richter, how soon after thawing the platelets did they perform these *in vitro* tests?

E. Richter: Our results confirm that there was no correlation between *in vitro* function after thawing and survival *in vivo* using a ^{51}Cr labelling.

J.W. Armitage That then raises the question: How soon after the platelets were thawed, did you do the *in vitro* tests. Was it one hour after thawing or straight after thawing.

E. Richter: The *in vitro* tests were done after thawing and incubation for 1 hour at 37°C.

J.W. Armitage: That raises the question: What is the point of any of the *in vitro* tests for platelets at the moment. If the tests that have been used on platelets *in vitro* at the moment have no correlation whatsoever with *in vivo* function and survival or haemostatic effects then why bother with the *in vitro* tests; what are they telling us about the platelet?

E. Richter: There was no linear correlation, which means that either there was poor *in vitro* survival but still some increment and on the other hand

there was much better *in vitro* survival but less increment. However, you have to take into account that in the patient there are some different conditions like fever, active bleeding or as I told some immunogenic activities so that you loose more platelets. This is the problem of the increment as a parameter of good *in vivo* survival, for instance.

J.W. Armitage: Well, this partially answers my question. Clearly, when you are doing studies on cell preservation, you cannot do all your function

tests *in vivo*. You have some sort of *in vitro* screening assay for sorting out differences between the many variables involved. We have to find a relevant *in vitro* test that actually bears some relation to the physiological function of the cell *in vivo*.

S. Holme: In our laboratory we have looked at the relationship between *in vitro* assays and post-transfusion survival studies in approximately 170 studies. This is with liquid storage of platelet concentrates. The *in vitro* parameter that we feel is the best predictor of *in vivo* viability is the discoid morphology. Another good predictor is the maintenance of respiratory activity, the ability to utilize oxygen. Furthermore, the maintenance of adenine nucleotides or ATP levels seems to be a good predictor for viability as well. Hypotonic shock, on the other hand, is not such a good predictor of viability. Neither is LDH release, nor release of β-thromboglobulin, which are commonly used *in vitro* assays.

C.F. Högman: Sometimes there is the discussion on how sure can you be that the platelets recovered *in vivo* are really well functioning. For instance the release of α-granula which you can measure by β-thromboglobulin or platelet factor 4 analysis influence the *in vivo* platelet function, but perhaps in such a way that it is very difficult to test.

Dr. Holme, what do you think about bleeding time, since there are now possibilities to perform *in vitro* bleeding times?

S. Holme: There are very few laboratories that I know of which are able to do infusion studies in thrombocytopenic patients and do bleeding times and aggregation studies to test the platelet function. For instance, it is well known that platelets stored in the cold aggregate much better than platelets stored at room temperature. Also platelets stored in particular synthetic media often maintain their aggregability quite well. But, on the other hand, the viability is rapidly lost post-infusion with storage in cold or in specific synthetic media. So, as far as I know there is no good *in vitro* assay, that accurately predicts the haemostatic function of the platelets after infusion. That is why we need such a test.

C.F. Högman: The reason why I am raising this question is that even if you have circulating platelets and a normal half-time, you cannot be absolutely sure that these platelets are quite normal in their function. That is why I do think we should go further to look for methods by which this could be measured.

C.Th. Smit Sibinga: This is an extremely important point, specifically the devil's advocate question raised by Dr. Armitage. Indeed, it is extremely difficult to match and correlate the *in vitro* in the *in vivo* specifically in platelets. Above all we deal with a population of platelets of different stages in development and function. What Dr. Richter said, probably

may not have come over as it should. However, it matches our own information. In the *in vitro* situation, in the freezing of platelets as we have used this for autologous purposes, we found the hypotonic shock response the most convenient and the most simple technique which tells us at least something about the integral and membrane function of the platelets and the contractability; indeed it is a coarse test. Of course, you could do a whole array of tests on platelets but in general it does not add more specific information. The proof of the pudding is in the eating, that is in the haemostatic capacity. We have looked into that aspect very critically in comparison with allogeneic fresh platelets: The actual capacity in haemostasis and the need of patients in the time as compared to fresh platelets. That may provide some answer. But I would actually leave that for Dr. Mulder to report in her presentation.

P.C. Das: May I remind our audience that platelet function testing is a battle field which has been fought for the last 25 years. Last review is in Vox Sanguinis[1] 8 years ago. What Dr. Smit Sibinga said that the *in vitro* tests and function *in vivo* do not correlate well is correct. But, you see we are all looking for Plato's utopia. We must do certain tests. Particularly when you produce platelets from donor blood, there must be checks to assure that production is alright and that you are not damaging too much. There is no way one could abandon this principle. For example, Dr. Beaujean might tell you that many culture techniques (CFU-C's) have been reported in the literature. If you look at their numbers and plot them over days in which the bone marrow recovery takes place, they do not correlate very well. But that does not mean we should not do it, because when one manipulates vital organs like blood and blood products you are bound to do certain tests. There is no other alternative.

C.Th. Smit Sibinga: Adding to that; what of course we have done on purpose is careful *in vitro* study before using frozen-thawed platelets. We have not had the guts to infuse in patients without testing. The problem, however, in the *in vivo* model is that in the autologous situation it is absolutely impossible sofar to include a stable, thrombocytopenic patient population, because these patients depend on the platelets in their situation. However, they have fever, or an enlarged spleen or they have an active bleeding tendency. All these conditions also have an effect on the survival of platelets, but what we found again is a good haemostatic capacity as compared to infusion of fresh allogeneic platelets and that was the actual proof of our pudding.

1) *In vitro* measurements of stored platelet concentrates. Vox Sang 1981;40 (suppl.1).

A. Brand (Leiden, NL): I totally disagree with this statement. We infused for 20 years beautiful freshly prepared platelets, within 4 hours after donation. We prepared them in bottles with extra ACD and we could infuse via central lines. We could take spleens out, we could do surgery with perfect bleeding times and when we did aggregation and other assays with these types of platelets they were horrible, lower than 20%. Now we store platelets for 5 days and we tried to get the same bleeding time corrections to do the same surgery. Aggregation studies and all kind of other platelet function studies are far better than in our fresh platelets, but the bleeding time corrections are worse.

C.Th. Smit Sibinga: In fact we do not disagree at all! However, we are talking about totally different approaches and regimes.

IV. ADVANCES AND CLINICAL APPLICATIONS

CLINICAL APPLICATION OF CRYOPRESERVED STEM CELLS AND PLATELETS IN A BONE MARROW TRANSPLANT PROGRAM

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Bone marrow transplantation (BMT) has become a rapidly emerging adjunct to the treatment of patients with leukemia, lymphoma and selected solid tumors. On a lesser scale BMT is being utilized in a number of non-malignant hematologic conditions such as immunodeficiency, aplastic anemia, severe radiation-induced bone marrow damage, thalassemia and other genetic disorders.

In allogeneic BMT the bone is derived from a healthy HLA-compatible donor. In autologous BMT (ABMT) the patient acts as his/her own donor: before the start of marrow ablative treatment, stem cells are collected and cryopreserved and then reinfused to provide hematologic recovery. Although bone marrow is a rich and common source of stem cells, it is feasible to collect stem cells from the peripheral blood by leukapheresis. This is usually performed in the recovery phase from conventional chemotherapy when the number of circulating progenitor cells is increased [1,2].

In clinical Phase I cancer trials, ABMT has permitted to explore dose-escalation of chemotherapeutic agents till the maximum tolerated dose for extra-medullary toxicity. Furthermore, in Phase II trials BMT has offered the opportunity to applicate high-dose chemotherapy aimed at identifying responses and curative possibilities in certain malignancies relapsing after or refractory to standard antineoplastic treatment. In the clinical situation the curative potential of high-dose chemotherapy combined with allogeneic BMT or ABMT has been proven in some hematological malignancies [3,4]. This therapeutic approach is in the treatment of advanced or disseminated solid tumors still in the experimental phase.

This review will outline the clinical aspects of application of cryopreserved autologous hemopoietic stem cells and platelets as adjunct to the treatment with intensive chemotherapy from a group of 96 adult patients with advanced or disseminated solid tumors in our institute. All patients had received prior chemotherapy for breast cancer (n=34), non-seminomatous germ cell tumors (NSGCT, n=18), small cell lung cancer (SCLC, n=18), epithelial ovarian cancer (n=20) and other solid tumors

(n=1 gastric cancer , n=1 colon cancer, n=1 adenocarcinoma of the lung, n=1 renal cell cancer, n=2 malignant melanoma).

Bone marrow collection *in vivo*

Bone marrow collection is usually performed under general anesthesia [5]. We and others reported earlier on the feasibility of bone marrow harvest without general anesthesia [6-8]. In summary: bone marrow was collected 101 times from the posterior iliac crest at least one hour after premedication with 100 mg meperidine i.m. and 20 mg diazepam i.m. Local anesthesia was achieved with 35 ml lidocain 1% s.c. The mean time of the whole procedure which was performed on an outpatient basis was 80 minutes. The acceptance by the patients was in general very good. Four times short lasting hypotension and four times bradycardia with excellent response on atropine occurred. Vomiting due to meperidine could be avoided by keeping the patient fasting.

Transfusion associated graft vs host disease in ABMT

To compensate for the aspirated volume (mean 1.07 liter), during and after the harvesting 3 units of leukocyte poor red blood cells were transfused. In 4 out of 34 patients treated with intensive chemotherapy and reinfusion of autologous marrow we found histological and clinical evidence of graft vs host disease (GVHD) [9,10]. The time course of events suggested that blood given during the bone marrow harvesting could have contaminated the marrow with allogeneic cells, and the bone marrow was the first blood product given to these patients in their aplastic period. Consequently all allogeneic transfusions were irradiated with 15 Gy to eliminate the engraftment potential of the infused lymphocytes [11]. Since then in 62 procedures no such complications have occurred.

Bone marrow surveillance of tumor cells

Bone marrow involvement with tumor cells is a major problem in the leukemia's and lymphoma's. In certain tumors such as SCLC, breast cancer and melanoma, bone marrow micrometastasis occur. We saw for instance in a group of 56 patients with advanced or disseminated breast cancer 10 patients with positive marrow aspirates prior to remission induction therapy. Recently it was shown that many of the bone marrow micrometastases in breast cancer patients are the result of shedding of cells from the primary cancer and that a proportion of these tumor cells is not viable *in vivo* [12]. Therefore, the question can be raised whether infusion of small numbers of neoplastic cells in the autograft is of clinical relevance. Marrow contaminated with viable tumor cells would presumably produce a relapse pattern that is independent of the distribution of pretreatment tumor. Present experience in ABMT for lymphoma's has emphasized the importance of original residual disease. Furthermore, while *in vitro* purging of the procured bone marrow with biophysical, pharmacological or immunological methods has been successful in the

laboratory [13,14], it remains controversial in the context of clinical trials [15]. The benefit of purging may be brought to light when treatment with high-dose chemotherapy and ABMT will show long-term control of presenting metastasis in a substantial number of patients with cancer.

In our ABMT program, patients were selected for absence of bone marrow metastases by bilateral biopsies and smears from the iliac crests and by tumor clonogenic assays of the harvested marrow. So, patients with bone marrow involvement at presentation, were re-examined after remission induction chemotherapy. One patient with breast cancern showed after autografting a relapse pattern different from sites of previous extensive disease, suggesting out-growth of reinfused tumor cells.

Bacteriological surveillance

Bacteriological cultures of marrow samples were routinely performed before cryopreservation and after thawing. Evaluation of 83 harvesting procedures (a total of 178 bags) showed 11 positive cultures originating from nine patients. Of five of these patients 8 additional samples of bone marrow were cultured. In only one patient 2 samples were positive. Of 311 samples of frozen and thawed bone marrow (of 75 patients) 12 examples were positive from 8 patients. It can be concluded that accidental contamination is frequently encountered in bacteriological culturing of bone marrow.

Quality surveillance

A minimum number of 2×10^8 nucleated cells per kg body weight was collected during the harvesting procedure. Recovery after preparation *in vitro* was approximately 50%. Granulocyte macrophage colony forming units (CFU-GM) were determined in a two layer agar system using bone marrow samples obtained during collection, after apheresis, before freezing and after thawing, to evaluate the procedure in 6 patients. The CFU-GM were routinely measured in harvesting marrow of all patients. In our patients mean numbers of CFU-GM transfused were 40.09×10^3/kg body weight (SD 36.80) and a mean of 1.41×10^8 nucleated cells/kg body weight. Two patients suffered a very delayed hemopoietic regeneration. In one of these patients no CFU-GM at all could be cultured from the bone marrow, in the other patient even 45 CFU-GM per 10^5 nucleated cells were cultured. No clear correlation could be found between the number of CFU-GM infused and the duration of leuko- and thrombocytopenia in all other patients.

The duration of leukopenia and thrombopenia varied with the high-dose regimen used. For example with the combination regimen of cyclophosphamide and etoposide the median duration of leukopenia (less than 1.0×10^9/l) was 15 days [range 11–22] and the median duration of thrombopenia (less than 40×10^9/l) was 14 days [9-28]. With the high-dose regimen of cyclophosphamide and mitoxantrone the median

duration was 22 days for both leukocytopenia and thrombocytopenia. Active herpes simplex virus infection in the posttransplant period was found to have no influence on the speed of white cell and platelet recovery [16], as has been suggested by others [17].

We do not know whether hemopoiesis recovers from residual stem cells that have survived the intensive chemotherapy or from the reinfused bone marrow or both. However, we consider it hardly ethical to omit ABMT in a randomized study.

Timing of high-dose chemotherapy and ABMT in solid tumors

In the experimental setting most of the experience with high-dose chemotherapy and ABMT was gained in patients with relapsing or refractory tumors after prior conventional anti-neoplastic therapy. Despite heavy pretreatment the response rate in these patients is usually high. However, partial responses outnumber complete responses and most remissions are only brief. Cure, or at least longstanding disease free survival, is only possible after achievement of a complete remission. In hematologic malignancies factors associated with a favorable outcome are: transplantation in first remission, reduced tumor burden and demonstrated responsiveness to prior chemotherapy [3,18,19]. For leukemia in relapse, autografting has not been rewarding and few centers persist with this approach.

Also for solid tumors, the status of first remission is probably the situation in which there is the best opportunity of effecting a cure, as the tumor is by definition chemosensitive and occult disease is more readily treated than bulk disease. Also, in our experience with patients treated for bulky ovarian cancer and germ cell tumors, the chance to achieve a complete remission and a long-term survival was low [20,21], whereas chances improved in the situation of minimal residual ovarian cancer [20] and breast cancer in complete remission [22]. Appropriate radiotherapy (or surgery) after transplantation may further enhance the curative potential of intensive chemotherapy and bone marrow rescue.

Timing of bone marrow reinfusion

The moment of bone marrow reinfusion depends on the disappearance from the plasma of toxic chemotherapeutic agents and their metabolites. The pharmacokinetic behavior of the different drugs used in the high-dose regimen is therefore of importance.

The antitumor drugs and total doses used in our clinical trials were: cyclphosphamide 7 g/m^2, etoposide 0.9-3 g/m^2, teniposide 500 mg/m^2, mitoxantrone 30-75 mg/m^2, melphalan 180 mg/m^2, dacarbazine 1.2 g/m^2 and carboplatin 750 mg/m^2. Melphalan and cyclophosphamide are rapidly cleared from the plasma [23], etoposide is still detectable after 6 days [24]. Pharmacokinetic studies showed that after

high-dose mitoxantrone and carboplatin bone marrow reinfusion can be done respectively 96-120 and 48-72 hours after the last dose [25,26]. High-dose chemotherapy had been given in our patients on day 1, 2 and 3. Reinfusion of autologous bone marrow was performed on day 7. The cryopreserved marrow was rapidly thawed in a waterbath at 40°C. Prior to bone marrow infusion via a central venous (Hickman) catheter prednisone and clemastine i.v. were given to prevent allergic reactions to the cryoprotectant dimethyl sulfoxide. Despite this premedication one patient suffered an anaphylactic reaction and cardiopulmonary arrest during the reinfusion procedure, but was successfully resuscitated.

Morbidity and mortality of high-dose chemotherapy and ABMT

The magnitude and duration of morbidity of ABMT is substantially lower than that of allogeneic BMT, mainly due to the absence of graft vs host disease. However, transfusion associated GVHD was encountered after autografting in 4 of our patients [vide supra].

Apart from GVHD, morbidity and mortality is caused by
- severe infections due to prolonged and deep granulocytopenia;
- bleeding tendency due to prolonged thrombocytopenia;
- emergence of extramedullary manifestations of drug toxicity which were not encountered at conventional doses.

The mortality from ABMT in solid tumor patients in phase I and early phase II studies ranges from 5-25% [27]. The overall mortality in the immediate posttransplant period was in our institute 10% and death was caused by infectious complications.

Bacterial infections were microbiologically documented in 57% of the patients including bacteremia in 80% of these cases. These infections were caused by Gram-negative micro-organisms in 20%, by Gram-positive cocci in 72% and by yeasts or fungi in 8%. The low incidence of Gram-negative bacterial infections can probably be attributed to selective decontamination of these bacteria from the gut by the prophylactic use of antimicrobial agents that do not disturb the anaerobic microflora, which is responsible for the colonization resistance. Three regimens were evaluated consisting of polymyxin B combined with either oral neomycin, or parenteral temocillin or oral cotrimoxazol. All three regimens were effective in decolonization of the gut. Other practical measures for infection prevention are nursing in a single hospital room and providing of sterile food. The high incidence of Gram-positive infections (50% of all ABMT patients), mainly caused by bacteria from the group of viridans streptococci, and coagulase negative staphylococci may have been enhanced by the oropharyngeal mucositis induced by cytostatic chemotherapy and the indwelling Hickman catheter. The increasing prevalence of Gram-positive septicaemia has also been observed by others [28]. For the moment the problem how to effectively

prevent Gram-positive and fungal infections remains unsolved. Several studies have demonstrated the efficacy of laminary flow isolation for prevention of infection, however it does not improve survival and is not cost effective. It is only recommended in institutions where aspergillus is endemic [29]. At present more gain may be expected from the use of hematopoietic growth factors to shorten the leukopenic period than from prophylaxis with local and/or systemic antibiotics or isolation in laminary flow.

Viral infections may also cause morbidity and mortality in ABMT patients. Infections with the herpes simplex virus (HSV) was most common, due to reactivation in 63% of patients who were seropositive for HSV antibody before transplantation. Primary HSV infections did not occur. Acyclovir was very effective in preventing reactivation of HSV infections.

In contrast to the experience in allo-BMT, where interstitial pneumonia associated with cytomegalovirus (CMV) is the most common and severe problem after engraftment [30], infection with CMV without pulmonary symptoms occurred only in 3% of our ABMT patients despite the fact that the virus was latent in half of the patients at the start of intensive chemotherapy.

Possible reasons for the difference in incidence of CMV infection in ABMT and allo-BMT could be the difference in recovery in immunocompetence [31]. A shorter period of immunocompetence can influence frequency and severity of CMV infections following BMT as was shown in the difference between T-cell depleted and non T-cell depleted marrow recipients [32].

Primary CMV infections are usually caused by infected grafts or blood products. This has resulted in a tendency to restrict transfusion support of immunodeficient CMV negative patients to blood products supplied by CMV negative donors. Another approach consists of the transfusion of autologous blood products. Viral transmission and the need for CMV negative blood products was undoubtedly reduced by transfusion of cryopreserved autologous platelets in our patients.

Bleeding prophylaxis

Severe thrombocytopenia exists during a period of 15-28 days, starting approximately one week after the beginning of high-dose chemotherapy.

Allogeneic thrombocytes are most often used for bleeding prevention. However, autologous platelets eliminate the risk of transfusion related viral disease, GVHD and immune reactions. We studied the feasibility of bleeding prophylaxis using autologous platelets, cryopreserved prior to high-dose chemotherapy. In nearly half of the 43 patients who were studied, the thrombocytopenic period could be bridged with four donations of their own platelets. In the other half of the patients either more than 4 transfusions were required, or inaccessible veins or

insufficient time for platelet apheresis precluded the sole use of autologous thrombocytes.

No bleeding episodes occurred following the administration of prophylactic autologous platelet transfusions, so in that respect they were as active as fresh single donor allogeneic platelets. The one hour increment for allogeneic thrombocytes was twice as high as for autologous thrombocytes, but the interval between transfusion in days was the same. So most probably autologous platelets possess a longer *in vivo* life span than allogeneic platelets. The washing step for removal of the cryoprotectant of the autologous platelets can be omitted without adversely affecting the bleeding prophylaxis capacity and also without introducing immediate or late complications due to intravenous administration of the cryoprotectant (5% dimethylsulfoxide) [33].

Human growth factors

Autologous bone marrow rescue is given after high-dose chemotherapy to reverse bone marrow suppression and accelerate recovery of hematopoiesis. Despite intensive supportive care the morbidity and mortality in the immediate posttransplant period is high due to complications caused by longstanding granulocyto- and thrombocytopenia.
The recent availability for clinical use of a series of recombinant human growth factors (HGF) might make autotransplantation safer.

The *in vivo* administration of HGF before the collection of bone marrow or peripheral stem cells may enhance the number of progenitor cells [34,35]. This could lead to accelerated engraftment, provided there is a relationship between the number of progenitor cells and the time needed for hematopoietic recovery. However this relationship has been demonstrated only for low numbers [36]. On the other hand the *in vivo* use of HGF might be important in maintaining adequate numbers of precursor cells in *ex vivo* purging procedures.

Clinical trials using HGF after stem cell reinfusion showed a shortening in the duration of leukopenia [37] and resulted in a decrease in the incidence of infections [38].

It remains to be seen which growth factors will drastically shorten the period of thrombocytopenia.

It has been speculated that HGF may replace autotransplantation in the future because some hematopoietic stem cells probably survive most of the intensive chemo(radio)therapy regimens. The question can be raised if patients will survive this procedure. For the moment the risk of a very delayed or absent hematopoietic recovery outweighs the burden of bone marrow harvesting, cryopreservation and reinfusion.

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CLINICAL EFFICACY OF CRYOPRESERVED BONE

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Introduction

The first published case of bone grafting was performed in 1810 by Van Mecharen in Amsterdam. Although for some time autologous grafts have proven to be effective, allografts have often been used since 1879, when Mac Ewen treated a patient with humoral pseudoarthrosis with a fresh graft from an amputated limb. Since then, authors have reported many long-term positive results [1-8].

From 1976 on we have experience in bone banking, that has allowed large numbers of allografts to be done [9]. Most advantages of a bone bank are obvious. It is not only convenient for the surgeon but also pleasant for the patient [10]. Bone banking permits provision of bones and allows operations which could not be realized without it [11,12].

Since 1983 in Marseille, 790 femoral heads, 76 long bones and 8 joins have been cryopreserved and subsequently used.

The conditions for exclusion of a patient from donating bone are:

1. Infections: Recent history of active systemic infection. History of active hepatitis, tuberculosis, leprosy, malaria or syphilis. Acquired immunodeficiency syndrome.
2. Malignant disease, except healed primary basal cell carcinoma.
3. Autoimmune disease.
4. Chronic steroid therapy.
5. Irradiation of tissues to be collected.

Tests before tissue donation and cryopreservation are the same as for routine blood donation: HBsAg, HBcAb, ALT, HIV Ab, CMV Ab and VDRL.

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3. CHU Lapeyronie Montpellier, France

Figure 1a. Radial head piece no 1: 10% DMSO, cooling rate 1°C/min.

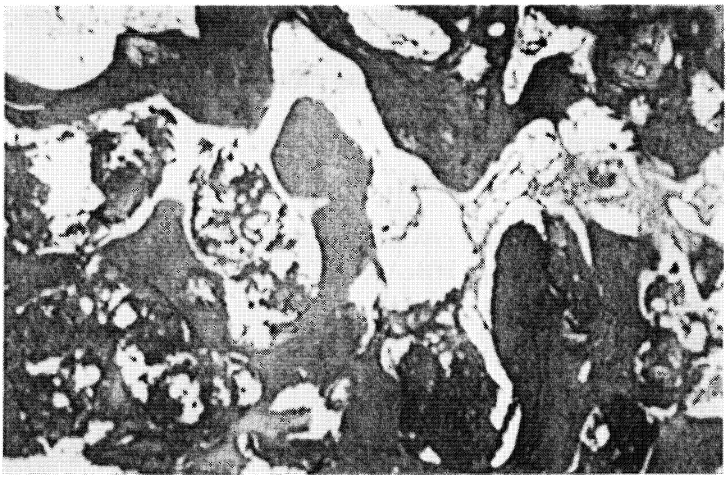

Figure 1b. Radial head piece no 2: 15% glycerol, cooling rate 1°C/min.

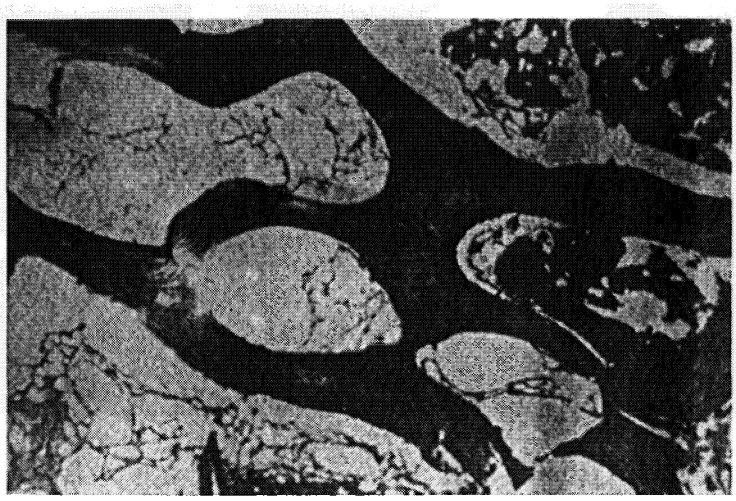

Figure 1c. Radial head piece no 3: 10% DMSO, cooling rate 35°C/min.

Materials and methods

Bone is collected in the operating room and put in Gambro bags under sterile conditions. The bone is then cooled to 4°C and sent to the Blood Centre within 24 hours after collection. An antibiotic solution is added. The cryoprotectant solution used is a mixture of human serum albumin and 10% DMSO. Freezing is accomplished at a cooling rate of 1-2°C/min down to –40°C and 5°C/min down to –140°C. The tissue is stored either in the liquid or the vapour phase of N_2.

The bone thawing is done rapidly in a waterbath at 42°C. DMSO is removed by washing. The sterility of the entire procedure is checked by bacteriological control.

The choice of DMSO and the cooling rate was made in 1983 after studies on the efficacy of cryopreservation of bone. In this study a radial head was divided into three pieces:

a. frozen with 10% DMSO at a cooling rate of 1°C/min;
b. frozen with 15% glycerol at a cooling rate of 1°C/min;
c. frozen with 10% DMSO at a cooling rate of 35°C/min.

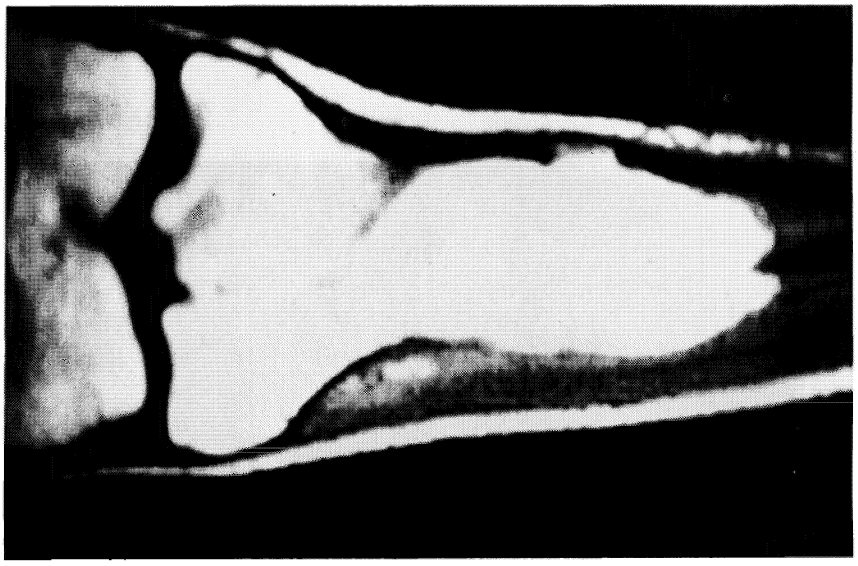

Figure 2a. Upper part of the tibia. Radiography of giant cell tumour.

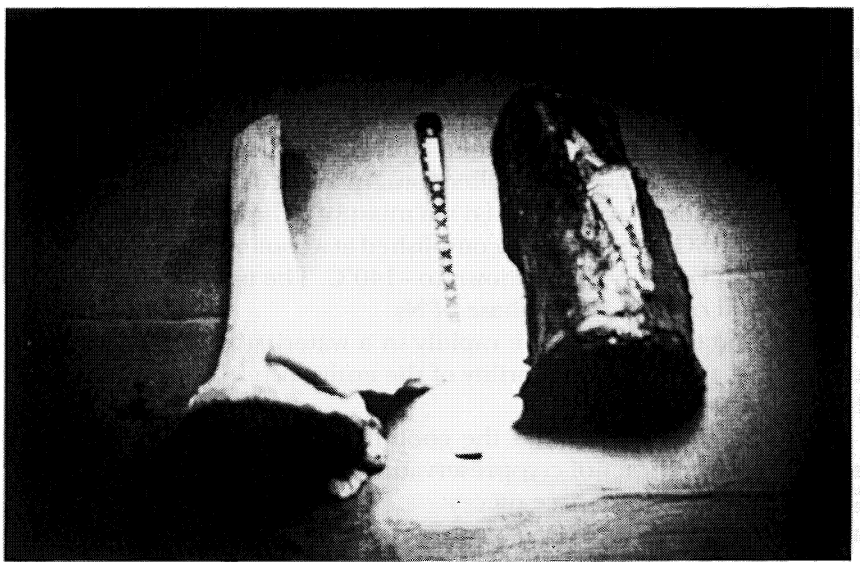

Figure 2b. Left: Shaped allograft before implantation. Right: Excised tumour.

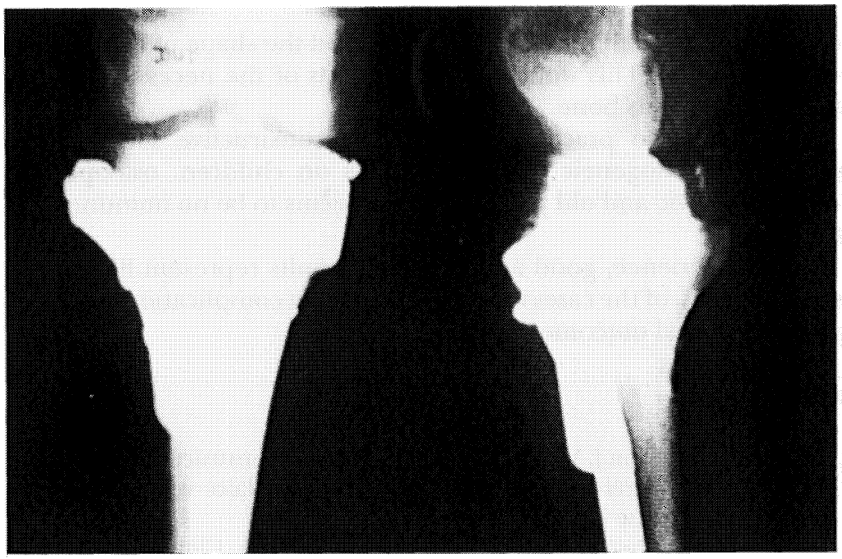

Figure 2c. Follow-up radiography 24 months after implantation.

Results

The results of the cryopreservation experiments are shown in Figure 1. The best results were obtained by a solution with 10% DMSO and a cooling rate of 1°C/min according to the vascular integrity (Figure 1a). Vascular integrity is important for repopulation of the bone both by recipient and graft cells.

Between 1983 and 1988 the orthopedic surgical team transplanted 373 cases of fresh and frozen allografts and 64 cases of massive allografts. One such successful outcome is illustrated in Figure 2, showing an adult patient receiving a bone allograft following excision of a tumour of the upper part of the tibia and its follow-up 2 years later (Figure 2c).

Complications

Complications related to the use of bone allografts have been described in great detail by several authors [3,5,8]. In fact, the complications determine the limits of this transplant surgery. With the exception of complications due to local tumour recurrence, several other complications are inherent to this type of surgery: infection, fracture, joint instability, necrosis and arthrosis, lack of consolidation. These complications represent 10-15% of the cases.

Conclusion

There is virtually no limitation on the size and the shape of the bone to be cryopreserved. This would relieve patients of the necessity of their own iliac cancellous bone.

It is possible to practice orthopedic reconstructive surgery with cryopreserved allogeneic bone and joints on children, osteoporotic patients, paralytic and old patients. There seems to be no immunologic response.

In our experience, good and excellent results represent 85% of all cases, while 90% of the cases who recovered from complications did have a good functional outcome.

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VITRIFICATION AS AN APPROACH TO ORGAN CRYOPRESERVATION: PAST, PRESENT, AND FUTURE

G.M. Fahy*

The concept of preserving organs in a viable condition outside of the human body dates at least from 1812, when LeGallois first proposed that after death of the body the human head (and therefore the individual) could be kept alive by removing it and supporting it by normothermic artificial machine perfusion [1]. History, however, has gone instead in the direction of hypothermic organ preservation, which seems to be a better option for both biological and economic reasons. The subject of this paper still lies in the future, and that is "cryothermic" preservation, i.e., preservation of organs at temperatures below –100°C. The primary advantage of this approach, should it prove to be possible, is that preservation times should become indefinite at such temperatures, thereby opening up many new and significant opportunities in transplantation medicine. This paper reviews the many steps toward the goal of organ cryopreservation by vitrification which have been taken since the beginning of this field in 1980, and also provides a very brief sketch of the rather ironic history of this area of research.

The past

Premodern impressions and developments

The possible value of cryopreservation was recognized early by such individuals as Robert Boyle [2] and John Hunter [3], who were interested in preserving quite complicated biological systems (such as themselves!). However, Boyle died as a result of his own exposure to subfreezing temperatures, and Hunter became discouraged when everything he froze died. Unfortunately, the experience of later workers was not much different. There was in fact some basis for Luyet's conclusion in 1937 that ice formation is not kind to living systems and ought to be avoided if possible [4].

* Supported in part by grants (GM 17959 and BSRG 2507 RR05737) from the National Institutes of Health, Bethesda, MD.

Luyet felt that it might be possible to cool small living systems at ultrahigh rates so as to preclude ice formation and achieve instead a glassy or vitreous state[4], wherein molecular translational motions have been arrested without structural reorganization of the liquid [5]. Thus was born the idea of vitrification, but not the idea of vitrifying organs, which was unthinkable at the cooling and warming rates required by Luyet's approach.

Vitrification of organs: Historical antecedents since 1949

It was the modern introduction [6] and general understanding [7] of cryoprotective agents which provided the first hope that organs might be preserved at low temperatures. Research spearheaded primarily by A.U. Smith and her colleagues showed that glycerol and other cryoprotectants could protect just about every conceivable living system from freezing and thawing damage provided the agent was applied correctly and the system was cooled and warmed properly [8]. It seemed that nothing could fail to be cryopreservable if only a few simple rules were observed, and it was not long before efforts were made to freeze not only isolated organs such as the kidney [9] but also entire mammals [10].

These efforts were for the most part doomed to failure due to the mechanically damaging effects of ice [11], but this was far from clear at the time due to numerous examples provided by certain exceptional complex biological systems which were known to survive after massive distortion from ice crystals [12-14]. What appears at present to be basically the correct general approach to organ cryopreservation was introduced independently and simultaneously by John Farant [15] and by Charles Huggins [16] in 1965 in two highly insightful and potentially revolutionary papers. Both men proposed using very high concentrations of cryoprotective agents, and both proposed doing so for the wrong reasons (i.e., to protect against electrolytic mediated injury rather than to protect against mechanical injury from ice). Neither had any awareness that the concentrations of cryoprotectant they proposed using could have allowed them to achieve, in 1965, vitrification of the unusually hardy systems they worked with if only they had cooled these systems to moderately lower temperatures.

Fortunately for organ cryopreservation, Luyet had by this time abandoned vitrification of unprotected samples as hopeless [17,18], and his research had ultimately turned to the antifreeze properties of cryoprotective agents. This research provided the primary key to organ cryopreservation by vitrification (i.e., the realization that high concentrations of cryoprotectant could permit vitrification even at very slow cooling rates), but neither Luyet himself nor any of his contemporaries realized it. Even more ironically, Luyet never consciously applied his insights on cryoprotectant action to vitrification of even tiny biological

systems, yet succeeded almost accidentally at erythrocyte vitrification without realizing the full significance of this accomplishment [19].

The origin of vitrification as an approach to organ cryopreservation

Much of the development of this idea came as a result of attempts [20,21] to implement Farant's technique [15] of progressively increasing dimethylsulfoxide concentration as the temperature was lowered. These studies showed that concentrated dimethylsulfoxide was too toxic to be useful and also that any attempt to vitrify this or any other cryoprotectant solution inevitably led to fracturing. Freezing, on the other hand, was found to cause mechanical disruption of both kidney slices [21,22] and entire dog kidneys [Fahy, Goldman, and Meryman, unpublished results]. An important step, however, was the development of carrier solutions which could maintain kidney tissue viability near 0°C for the several days which might be required for organ perfusion according to Farrant's technique [23,24].

An important turning point was the still-unpublished observation of Rajotte and McGann that dog kidney slices could tolerate exposure to total solute concentrations as high as 60% w/v at 0°C or above. It was soon possible to show that similar solutions could be stored for days and even for over a week at –79°C in a supercooled conditions [Fahy, unpublished observations], opening up the possibility of at least short term storage at sub-zero temperature. However, it was not possible to duplicate the dog kidney success using rabbit kidney slices [Fahy, unpublished results], and it was clear that it was critical to reduce the solute concentrations required for supercooling in order to reduce toxicity.

It was at about that time that Pierre Boutron published his first results with the miracle solute, propylene glycol [25], which seemed to be vitrifiable at concentrations near 40% w/w. The same concentration of dimethylsulfoxide was known to be reasonably nontoxic if added and removed in an optimum way [26]. It therefore again began to seem that prospects existed for avoiding solute toxicity while retaining supercooling and even vitrification ability.

This prospect inspired a reinvestigation of the question of fracturing, since vitrification was clearly a more desireable goal than mere supercooling. The answer at the time was provided by glass physical chemist Cornelius Moynihan, who stated that removing the container surrounding the vitrified system once the sample was below T_g should permit the avoidance of fractures during further slow cooling to –196°C [Moynihan, personal communication, 1980]. A single all-night experiment proved that he was correct [Fahy, unpublished results].

But vitrification of organs still seemed improbable without some other means of reducing cryoprotectant concentrations. It was at just that time that Allen Hirsh of our laboratory suggested the application of 2000 atm of hydrostatic pressure to a kidney so as to depress its freezing point to

about –21°C with no added cryoprotectant, thus making it possible to store the kidney longer than usual without any problems from cryoprotectant toxicity. Although Dr. Hirsh's experiment had been tried without success by several investigators in the past, it was immediately clear that the application of hydrostatic pressure should reduce the concentration of cryoprotectant required for vitrification.

This final key insight in combination with previous developments and indications that toxicity blocking agents existed for dimethylsulfoxide [27] provided the basis for the pursuit of organ vitrification.

Vitrification of organs: The present

What has happened in the nearly 9 years since the first stirrings of this research in late 1980? Are we any closer to a workable technique for organ vitrification? Is organ vitrification a realistic goal, or has the evidence proved discouraging? In fact, substantial progress has been made and this goal has appeared progressively more attainable as time has gone on. However, several serious problems have arisen within the past year.

This review will consider the advances which have been made in a hierarchical order. We will consider advances in the relevant physical chemistry of solutions, proceed through a consideration of cryoprotectant toxicity, and end with a brief examination of the current state of the art of vitrifying both small systems and organs. Since the latter requires specialized equipment, we will also note what equipment has and has not been developed for whole organ work.

Physics of vitrification and devitrification

We are beginning to understand why different solutes differ in their ability to promote vitrification. For example, the addition of methyl groups to a solute molecule such as ethylene glycol or acetamide significantly reduces the concentration required for vitrification (C_v) [28,29]. This is due largely to the electron induction effect, which causes the electron density about a hydroxyl or carboxyl oxygen to increase, i.e., which causes the oxygen basicity to increase [29]. A higher basicity induces stronger hydrogen bonding with water, hence a reduced crystallization tendency. It is remarkable that addition of a nominally nonpolar group such as $-CH_3$ causes more enhancement of water hydrogen bonding than the addition of a hydrophillic group such as $-CH_2OH$, yet this type of behavior is now well established. An example is addition of a methyl or a hydroxymethyl group to ethylene glycol: the former addition results in propylene glycol (1,2-propanediol), which vitrifies at a much lower concentration than the compound resulting from the latter addition, i.e., glycerol (1,2,3-propanetriol). An ethyl group provides a stronger inductive effect than a methyl group, and two separated methyl

groups provide a stronger effect than a single ethyl group according to NMR results [29]. It is not currently clear what the effects of other chemical groups such as $-NH_2$ or amide groups ($-CONH_2$) are or how the different chemical groups rank with respect to one another. Continuing studies along these lines may well allow us to better predict vitrification tendency and to design novel glass-promoting agents.

However, it is not just the presence of chemical groups which affects C_v and hydrogen bonding. 2,3-butanediol exists as three different stereochemical isomers, one of which, the meso form, forms hydrates which crystallise on cooling and even more rapidly on warming [30]. This example shows the importance of the spatial arrangement of chemical groups for water-solute hydrogen bonding.

Practical vitrification solutions contain not just one cryoprotectant but several. It is therefore of interest that it has proven to be possible to predict the approximate C_v of a mixture of cryoprotectants from the C_vs of the individual cryoprotectants within the mixture [31]. Recently, this generalization has been extended to the prediction of solution melting point (T_m), glass transition temperature (T_g), homogeneous nucleation temperature (T_h), and even devitrification temperature (T_d) [32]

Another physical phenomenon which has become better understood is the inhibition of ice crystal nucleation and growth by hydrostatic pressure [33]. In relatively dilute solutions (e.g., 20% w/w dimethylsulfoxide in water), the presence of the cryoprotectant results in a T_h near 2000 atm which is *above* the T_h of pure water at the same pressure [34]! The origin of this effect appears to lie in the reduced ability of high pressures to lower T_m when T_m has previously been lowered by the presence of the solute, and this phenomenon in turn is correlated with the difference between the properties of cryoprotectants, which enhance water structure, and the properties of electrolytes, which are "chaotropic" and disrupt water structure [33]. Fortunately, this negative trend due to cryoprotectants is reversed at higher concentrations, allowing pressure application to substantially reduce C_v. These insights open significant prospects for modifying vitrification solutions so as to reduce the inhibitory effects of the cryoprotectants, thus allowing C_v to be reduced by pressure further than is now possible.

Our most recent studies of vitrification tendency have explored the problems associated with cooling human-kidney-sized samples of vitrification solutions [35]. Although small samples of 50% w/w 1,2-propanediol vitrify readily, samples of 482 ml volume display multiple nucleation events, and crystal growth is appreciable in such samples. This occurs both because the cooling rate in large samples is necessarily limited and because what is improbable for a small unit volume becomes statistically more probable at much larger volumes. Most or all nucleation observed to date has been heterogeneous in character and may therefore be preventable. In addition, crystal growth rates are in general quite low in this vitrification solution, and it may become possible

to bring growth rates effectively to zero through the use of natural or synthetic antifreeze proteins or similar molecules.

The tendency for devitrification (the development of ice during warming of a vitrified solution) is also critically dependent on the growth rate of ice. In fact, devitrification of concentrated solutions is accounted for almost entirely by the growth of nuclei which formed during cooling at a temperature too low to permit crystal growth [36]. Unfortunately, very few data are available on ice crystal growth rates in biologically interesting vitrification solutions as a function of time and temperature, but this situation is changing [35,36]. Mathematical models of devitrification have acquired a high degree of descriptive power [36–38]. However, the mechanism of devitrification may change at very high concentrations or heating rates, introducing further complications [36].

Research had also shown that devitrification can be initiated in part by interfaces, i.e., the solution-air interface [39; Fahy, unpublished observations] and the interfaces created by fractures [36,39; unpublished results of R.J. Williams and P. Mehl]. The former source of nucleation can be avoided by covering the interface with a nonpolar phase such as isopentane [39; Fahy, unpublished results] while the latter source can be prevented by preventing the temperature from falling below about T_g minus 15°C [35].

The critical warming rate of vitrification solutions is the minimum rate necessary for the avoidance of significant devitrification upon warming. The predicted critical warming rates of Boutron have recently been revised downward significantly [38] and are now substantially in agreement with the earlier predictions of MacFarlane [37]. The result is that the critical heating rate is now believed to be substantially less than 1000°C/min for most vitrification solutions capable of being vitrified at cooling rates of about 10°C/min [28,40]. In fact, recent unpublished direct measurements indicate that critical heating rates of less than 100°C/min may pertain to many vitrification solutions, even after storage for several months just below T_g.

The final advance with respect to the physical chemistry of solutions is the direct suppression of devitrification by the use of naturally-occurring antifreeze proteins [41]. According to informal reports, C.A. Angell has succeeded in this endeavour, at least sporadically [J. Duman, personal communication]. However, as will be seen below, electromagnetic heating techniques appear to be capable of suppressing devitrification even in the absence of such proteins.

A fair summary of these andvances in the physical sciences upon which all prospects for succesful organ vitrification depend is that, with one exception, they all support the feasibility of designing and applying vitrification solutions and techniques to whole organs, especially in consideration of what has already been shown possible biologically as described below. The exception, the difficulty of suppressing nucleation and crystal growth in large, human-kidney-sized specimens, appears to

Table 1. Miscellaneous ineffective interventions.

Modifications of a standard vitrification solution or of the conditions of exposure to the vitrification solution which have been explored in an attempt to reduce the apparent toxicity of the solution for rabbit renal cortical slices, as judged by the ability of the slices to restore a normal intracellular electrolyte balance (K^+/Na^+ ratio) after removal of the cryoprotectants and incubation of the slices under conditions of active metabolism. This list complements a previous list of ineffective interventions [34], and thereby helps to reduce the number of possible mechanisms of toxicity and to direct attention toward other possible intervention strategies in the future.

Intervention	**Effect on viability**
Replace 3% PVP with 3% proline	more damage
Replace 6% PEG 8000 with 4% PEG 100,000	no change
Replace 6% PEG 8000 with 4% Pluronic F68	no change
Replace 6% PEG 8000 with 6% chondroitin sulfate	no change
Replace 6% PEG 8000 with 6% carboxymethyl cellulose	no change
Replace 6% PVP with 6% D(1)A	no change
Use 12% PVP and 35% D(1)AP_{10} instead of 6% PVP and 40% D(1)AP_{10}	no change or more damage
Dilute RPS-$2^=$ to 235mOsm	no change or more damage
Lower osmolality of RPS-$2^=$ to 235 and add glucose to restore total osmolality to 290mOsm	no change
Add dextrose to RPS-$2^=$ to give 400mOsm	more damage
Dilute RPS-$2^=$ to 161mOsm then add dextrose to give 400mOsm	more damage
Replace most glucose in RPS-$2^=$ with sucrose	no change or more damage
Saturate (bubble) solution with N_2	no change
Saturate (bubble) solution with O_2	no change
Raise redox potential with GSSG* or cytochrome c	no change
Delete reduced glutathione from RPS-$2^=$	no change
Add 0.001 M naloxone	no change
Add 20 mg% penicillin	possible slight improvement
add 10^{-7}M seleno-methionine, Na-selenite or se-purine	improved or not changed
Retain 2mM Mg^{++} (RPS-2^-) in presence of CPA	no change or more damage
Delete Mg^{++} from slice assay medium	no change
Raise exposure temperature to 10°C for concentration steps of 10% w/v and below	no change

The reference solution being altered in an attempt to reduce toxicity is 40% w/v D(1)AP_{10} + 6% PVP or + 6% PEG 8000 in an RPS-$2^=$ carrier solution. RPS-$2^=$ consists of 180mM dextrose, 28.2mM KCl, 10mM $NaHCO_3$, 7.2mM K_2HPO_4, 5mM reduced glutathione, and 1mM adenine.

* Oxidized glutathione

be a problem which at least can be addressed experimentally in a variety of ways.

Cryoprotectant toxicity

Research on mechanisms of and circumvention of cryoprotectant toxicity has progressed considerably, although challenges still remain. The toxicity of vitrifiable solutions has been sufficiently reduced to permit kidney slices to be treated with such solutions without an unacceptable loss of viability [34]. Further reductions in toxicity would enable higher concentrations to be used and would therefore minimize the need for high pressures, but there have been few if any significant gains since 1984. Table 1 lists some of the unsuccessful attempts to reduce toxicity.

Significant advances have been made with systems other than kidney. Single cell suspensions (erythrocytes, monocytes, and ova), embryos (rat, mouse, and cow), and tissues (especially islets of mouse, monkey, and man) have now been vitrified and recovered with a high degree of success [reviewed in 40,42]. In the case of mouse embryos, solutions have been found [43] which are considerably less toxic than the original VS1 solution used for vitrifying this system. Until recently, there were no examples of systems which could be vitrified but which could not be cryopreserved by freezing, but this is no longer true. Pig ova [44; Arav, personal communication), nonacclimated plant cells [45], schistosomes [46], and even *Drosophila melanogaster* [47] have now been successfully vitrified even though none of these highly diverse systems could be cryopreserved by freezing.

With respect to the toxicity of vitrification solutions,, it is becoming easier to conceptualize the mechanism. For example, it was recently shown that toxicity is not correlated with the tendency of these solutions to denature proteins. In fact, there is if anything a *negative* correlation [48]. This may be because so-called "kosmotropic" agents, i.e., water-organizing and protein stabilizing solutes, including cryoprotectants, can induce changes in membrane structure which may be damaging or lethal to living cells [48]. It has been shown that overstabilization of proteins by cryoprotectants can damage mitotic capability and therefore also be harmful [48].

One possible protein-related correlate of toxicity and its reduction is shown in figure 1, which illustrates the excess enthalpy of interaction between lysine and a variety of cryoprotectants. The excess enthalpy is represented as the second virial coefficient of the equation describing the contributions of all possible interactions to the overall observed excess enthalpy [48]. The excess enthalpy of interaction between dimethylsulfoxide and lysine is strongly endothermic or "thermochemically repulsive", and this phenomenon may be related to the toxicity of dimethylsulfoxide [27,48]. Mixing dimethylsulfoxide with less thermochemically repulsive

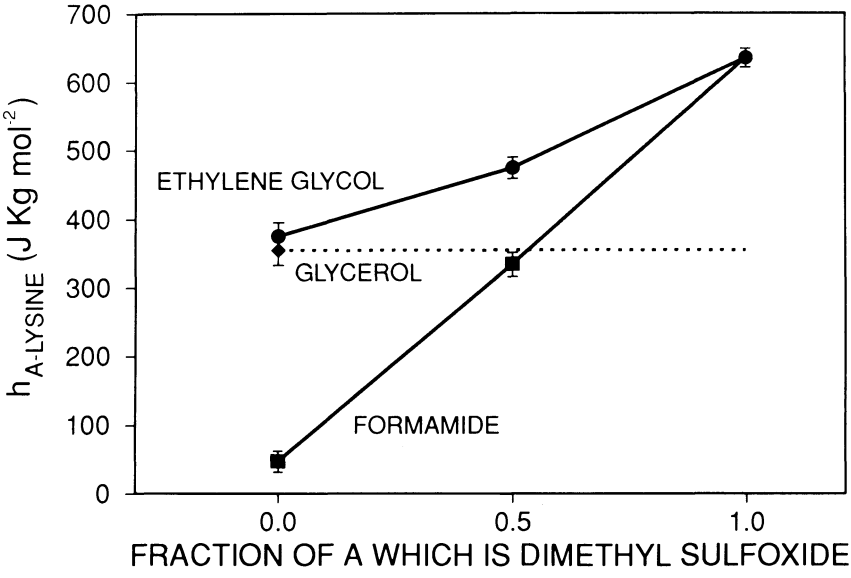

Figure 1. Enthalpies of interaction between cryoprotectants and lysine. The interaction between dimethylsulfoxide and lysine has been proposed to result in cellular toxicity [27], whereas glycerol is not harmful to proteins, and its interaction strength may therefore represent a level of interaction with lysine which is acceptable (horizontal dashed line). As indicated by the lines showing the effects of adding either ethylene glycol or formamide to dimethylsulfoxide, these interpretations are consistent with the fact that formamide is a more effective toxicity blocker for dimethylsulfoxide than is ethylene glycol. For further discission, [see ref. 48].

substances such as ethylene glycol or formamide reduces the magnitude of the enthalpy of interaction, but the former additive does not have the same efficiency as the latter. The presence of glycerol is known to be innocuous for most proteins, and its interaction with lysine is less endothermic than that of dimethylsulfoxide. It is therefore of interest that mixtures of formamide and dimethylsulfoxide achieve the same enthalpy of interaction as glycerol at a lower mole ratio of additive to dimethylsulfoxide than is possible in the case of mixtures of ethylene glycol and dimethylsulfoxide. This is in agreement with the experimental observation that formamide is a more potent toxicity blocker for dimethylsulfoxide than is ethylene glycol [28].

Progress in the control of toxicity and the design of vitrifying solution has now been sufficient at the level of tissues to justify direct experiments on whole organs.

Equipment development

The feasibility of whole-organ experiments has been dependent on the development of several new pieces of equipment. One key element, mechanical freezers capable of operation at –150°C, became commercially available in about 1986. These freezers permit storage of large vitrified objects, such as kidneys, at low enough temperature to permit stable long term storage but at high enough temperatures to prevent the fracturing which would be difficult or impossible to avoid during cooling to or warming from liquid nitrogen temperature.

The first attempts to create a computer controlled perfusion system for vitrification solutions were reported late in 1985 [49], and the device finally became operational near the end of 1986.

Pressurisation equipment for large organs is under development. A pressure vessel large enough to contain human kidneys and fitted with electrodes for electromagnetic heating within the pressure vessel is ready for testing.

The development of means for rapid and uniform electromagnetic heating within the pressure vessel has been conducted with the collaboration of Dr. Paul Ruggera, Center for Devices and Radiological Health, U.S. Food and Drug Administration. The resulting system [50], is capable of heating dog kidney-sized samples at rates of at least 100°C/min at the highest powers (<800 watts) so far explored. The observed heating capacity of about 20°C/min/100 watts/100 ml of solution appears to be independent of both applied power and sample volume, which implies that it may be feasible to warm human-organ-sized samples at the requisite rates in the near future.

Rabbit kidney vitrification: current status

Initial attempts to perfuse rabbit kidneys with vitrifiable media were reported in some detail in 1987 [51]. At present, a major obstacle to successful organ vitrification involves ill-defined damage to the perfused kidney that results from cooling below 0°C, a poorly understood problem which has been documented in some detail by Pegg and his associates [52,53]. It is important to note that Jacobsen [54] as well as earlier investigators [55,56] did not observe this "chilling injury", opening the possibility that this kind of injury can be circumvented by relatively subtle modifications of technique. Of significance is our observation that vitrification even of rabbit kidney slices is not entirely innocuous. Whereas a previous report of damage in rabbit kidney slices vitrified and warmed in VS1 [40] could be ascribed to devitrification, more recent experiments have shown that about the same amount of injury is observed even when devitrification is avoided [unpublished results], suggesting that chilling injury may not be primarily a vascular phenomenon but instead a direct insult to the organ parenchyma.

Chilling injury may not be the only remaining obstacle to successful organ vitrification. Although rabbit renal cortical slices can tolerate the high pressures required for vitrification [34,57], it is not yet known whether the whole organ is similarly resistant. Literature reports indicate that both kidneys [58,59] and hearts [60] can tolerate at least very brief exposure to pressures of the order of 1000 atm in the absence of cryoprotectants. Although tolerance would be expected to be greater in the presence of cryoprotectants [34,57], only direct experiments can determine whether this final challenge can be successfully met.

The future

Although vitrification and successful reimplantation of an animal kidney has yet to be achieved, success with single cells or small multicellular specimens has already been demonstrated and reports of success with increasingly complex tissues can be anticipated in the near future. There is a large variety of potentially transplantable tissues that awaits only improved techniques for preventing graft rejection to become clinically useful. Some of the most promising advances in transplantation immunology involve the induction of donor-specific tolerance through the transfusion of appropriately modified leukocytes from the donor. Vitrified cadaver allografts of the future may well be banked in conjunction with cryopreserved blood cells from the donor. The necessity for immunological preparation of the recipient over an interval of two or more weeks will mandate cryopreservation of the allografts. Although conventional cryoprotected feezing may be an acceptable method for some tissues where a degree of tissue injury can be tolerated, the inevitable mechanical damage from ice formation will place a restrictive limit on the use of freezing, particularly of highly vascularized tissues in which the vascular endothelium is particularly vulnerable to freezing injury. Vitrification avoids the problems associated with ice formation, cell dehydration and the control of freezing rates and thus should have many applications. Much of the challenge associated with vitrification now concerns the toxicity of vitrifiable perfusion solutions, but this is a relatively new topic, as yet poorly understood and with much opportunity for developments of practical importance. No persuasive evidence has as yet been presented to contradict the expectation that the cryopreservation of tissues and organs is indeed possible and that we are on the right track.

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TRANSFUSION, TRANSPLANTATION AND THE IMMUNOLOGICAL REVOLUTION

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The cryopreservation of living human tissues and organs for transplantation is the next major challenge for cyrobiologists. But all the ingenuity and success that could be wished for will be of little practical utility until the obstacles of allograft rejection have been overcome and non-life-saving transplants can be performed without the major hazards of present-day immunosuppression.

The advent of monoclonal antibodies and the development of the fluorescence activated cell sorter (FACS) have provided powerful tools for the dissection of the cellular immune system and immunology has now become one of the most rapidly developing basic research areas of the biological sciences. Among the clinical applications of immunological research, transplantation is a major beneficiary of this surge in activity. In particular, the mechanism for the induction of tolerance is now receiving the attention it deserves, both to understand the natural development of tolerance to fetal and self-proteins and to develop practical procedures to enable the transplantation of viable allografts.

There is no question but that some degree of tolerance to allograft can be induced by transfusions [1,2]. Improved response to kidney transplant in previously transfused patients is well established and many investigators have reported moderate to complete tolerance in animals as a result of prior transfusions of selected or modified donor strain blood products. However, the mechanism of this tolerance induction remains uncertain. An antibody against the Fc receptor following blood transfusion has been reported [3] as has the development of anti-idiotypic antibodies. Both suppressor T-cells and those capable of preventing an immunological response by other cells (veto cells) have been invoked but not as yet unequivocally demonstrated [4, reviewed in 5]. Current opinion appears to favor the concept of "anergy", a down-regulating of T-cells which can result from alterations in the quality of the

* Part of this study was supported by a grant from the G. Harold and Leila Y. Mathers Charitable Foundation and National Institutes of Health Grant BSRG 2S07 RR 05737.

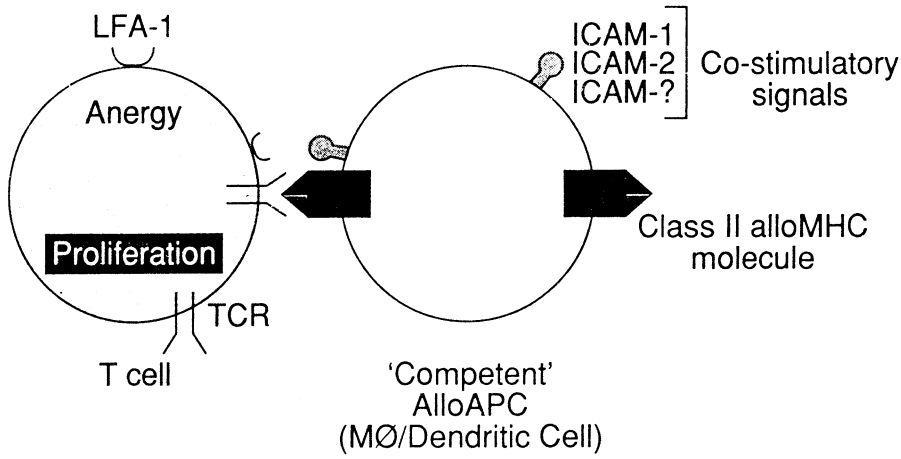

Figure 1. Mechanism of the cellular immune response.

signal transmitted by the antigen-presenting cell to the T-cell, and it is this concept that has been principally investigated in our laboratory.

This paper will deal primarily with the mechanism by which alloantigens initiate an immune response and the alterations of antigen-presenting cells (APCs) (also called accessory cells) that can convert an immunizing signal to a tolerizing signal. Current experimental evidence leads to the speculation that *in vitro* modification and transfusion of donor accessory cells may be sufficient to induce a state of T-cell anergy and permit subsequent stable acceptance of donor-specific allografts.

The vertebrate immune system is comprised of different interacting cells of bone marrow origin, the purpose of which are to seek out and destroy invaders threatening the homeostasis of the organism. Thymus-derived lymphocytes (T cells) are the major regulators of the immune vertebrate system. Their receptors interact with antigens which are presented in association with HLA molecules (in man), referred to in general as the major histocompatibility complex (MHC), which are expressed on the surface of specialized bone-marrow-derived, antigen-presenting cells [6]. These APCs do not present antigens directly, rather the antigens are internalized by the APC, degraded in intracellular acid compartments, and peptides derived from the antigen are expressed in conjunction with the MHC molecule and it is the combination of MHC and peptide that is recognized by the T-cell receptor [6].

There is good evidence that APCs are unselective, internalizing and processing proteins at random and expressing peptides derived from both native (self) and foreign antigens. It is the T-cells that are selective. Any individual T-cell clone is capable of recognizing only a single MHC-peptide combination but the number of individual clones is so large that most possible MHC-peptide combination can be recognized. In order to prevent an immune response to self proteins, T-cell clones capable of recognizing self antigens must either be "silenced" throughout their life span or eliminated [5]. Physical elimination of self-reacting immune cells occurs in the thymus during fetal development but it is questionable whether such "clonal deletion" occurs in the adult. The suppression of immune response to new antigens during adult life will surely require some other mechanism which well may be that operative during pregnancy, a natural allograft.

There are at least two subsets of mature T cells. CD4 (cluster determinant 4) T lymphocytes recognize antigenic peptides expressed in conjunction with class II MHC molecules (HLA DR, DP, DQ) on the surface of antigen presenting cells. Presentation of antigens to CD4 lymphocytes elicits secretion of regulatory cytokines such as interleukin-2 (IL-2), IL-4, gamma interferon, etc., which stimulate both antibody production and cellular defense mechanisms.

The other class of T lymphocytes, CD8 T cells, recognize antigenic peptides presented in conjunction with class I MHC molecules (HLA A,B,C) and proliferate after stimulation into effector cytotoxic cells capable of destroying specific targets. When stimulated to proliferate they become cytotoxic against cells bearing the specific class I antigen to which they have previously been exposed. Proliferation of both CD4 and CD8 cells requires stimulation by IL-2 which is secreted by the activated CD4 cell.

B cells are a separate class of lymphocytes which appear and mature in the bone marrow. Their surface receptor is a cell-bound immunoglobulin. When this receptor is stimulated and when the B cells are provided with certain lymphokines which are also secreted by CD4 T cells they secrete antigen specific antibodies.

There seem to be four major ways by which a normal immune response to alloantigen may be prevented:

1. antigens are not exposed to the immune system, as in corneal allografts;
2. antigen-reactive T cells are deleted from the repertoire;
3. a suppressor mechanism for the inhibition of antigen-reactive cells is invoked;
4. antigen-reactive cells are functionally inactivated (silenced).

Transplantation of tissues or organs at a site inaccessible to immunocompetent cells is usually impossible. Although there is solid evidence for physical elimination of self-reactive cells in the thymus during fetal de-

velopment, such a mechanism probably does not operate or is of minor significance in establishing tolerance to antigens in adulthood. The concept of separate suppressor T cells, lymphocytes that bear the CD8 phenotype and whose function is actively to turn off other immunocompetent cells in an antigen specific manner, has been challenged and remains controversial. Current sentiment is that suppression is mediated by both CD4+ and CD8+ T cells which, when appropriately signalled, are down-regulated rather than stimulated (functional suppression) or that suppressor cell phenomena are mediated by T cells which recognize idiotypes present on the receptors of anti-self cells, i.e. suppression is an anti-idiotype response [7,8].

There is accumulating evidence in the literature that functionally inactivated (tolerant) immunocompetent cells exist [9,10]. B lymphocytes exposed to multivalent antigens in the absence of T cells help enter a state of non-responsiveness to subsequent challenges. Tolerance in transgenic mice expressing allo MHC class I or class II molecules on pancreatic T cells has recently been described, supporting the expectation that peripheral T cell tolerance can also develop [9,11].

As summarized above, activation of CD4 cells to secrete the lymphokines necessary for the proliferation of effector cells, especially for the activation and proliferation of CD8 cytotoxic cells, requires an initial interaction between the CD4 receptor molecules and the MHC-peptide combination on the antigen-presenting cell. However, it is becoming increasingly evident that at least two separate signals are required for initiation of CD4 T-cell proliferation and the development of T effector cells [12,13]. Interaction between the clonotypic T cell receptor (TCR) and the MHC-peptide complex on the APC leads to the hydrolysis of membrane phosphatidylinositol in the T cell, resulting in the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG). Increased IP_3 leads in turn to a rise in intracellular Ca^{++} which, when combined with DAG, is responsible for the activation of proteinkinase C. These events lead to the expression of receptors for IL-2, but no proliferation is initiated since IL-2 is not secreted.

Expression of the IL-2 gene apparently depends on a second, "costimulatory" signal originating from surface molecules other than the MHC on specialized antigen presenting cells. More important, there is substantial evidence that, in the absence of costimulation and/or IL-2, T cells not only do not proliferate but become functionally tolerant (clonal anergy) and will not proliferate in response to a subsequent antigenic challenge, a phenomenon of major importance in transplantation immunobiology. According to this model, induction of transplantation tolerance would result from the presentation of the alloantigen (MHC-peptide) in the absence of the costimulatory signal [14]. Determining the nature of the alloantigens as well as the origin of costimulation is therefore of major importance and a prerequisite to an attempt to induce donor specific tolerance via clonal anergy.

During the last few years there have been several major developments in the understanding of alloantigen recognition by T cells. It was originally assumed that the immune response to an allograft was comparable to the response to a conventional antigen, i.e. that allo MHC antigens were directly recognized by recipient B lymphocytes, but needed to be processed and presented as fragments by host APCs in order to be recognized by the host T cells. It is now acknowledged that MHC molecules are designed to interact only with the clonotypic receptors on T cells and that MHC-peptide on donor APCs can be directly recognized by recipient 15].

A second major development is the description of the X-ray crystallographic structure of MHC molecules [16,17]. Both class I and class II MHC molecules serve as receptors for small protein fragments (peptides). Peptides bound to class II molecules are derived mostly from endosomal degradation of self-synthesized or extracellular proteins (antigens) while class I molecules bind to peptides which are located in the cytosol. The peptide binding site is bounded by two alpha-helical regions. Most polymorphic aminoacid residues lie on or between these helices and potentially affect both the shape of the binding site and epitopes exposed to the T cell.

There is experimental evidence that this site is always filled. In the absence of foreign antigen it will be filled with self peptides, and this suggests that the moiety recognized by alloreactive T cells is an MHC-peptide complex. Thus, to some degree, the high frequency of alloreactive T cells may be accounted for by the diversity of self – or antigen – derived peptide-MHC complexes that constitute the population of alloreactive molecules [18]. In the case of class II molecules this means that the full array of possible MHC-peptide combinations expressed on a transplanted donor antigen presenting cell would come not only from expression of self peptides but also from the expression of peptides derived from recipient proteins. While the former are expressed on resting donor APCs, the latter would be generated after donor APCs are allowed to process recipient proteins. In other words donor specific tolerance, established towards alloantigens expressed on donor resting cells may be sufficient to delay graft rejection but may be insufficient to allow permanent graft acceptance.

Our studies of the costimulatory signal have provided evidence that resting APCs do not express this signal at sufficient density to provide costimulation of T cells [19]. Such cells, inactivated by UVB irradiation or paraformaldehyde fixation, do not induce T-cell proliferation in the mixed lymphocyte culture and, *in vivo*, have been shown to lead to some degree of donor-specific tolerance in several experimental models [20]. However, according to our model, such cells will express only MHC-self peptide complexes and would be expected to induce only partial tolerance with delayed rejection since, following transplantation, the allo-APCs in the allograft will present an array of recipient peptides which,

in the context of alloMHC, will be seen as foreign by recipient T cells.

Full expression of all possible allodeterminants could perhaps be achieved by incubation of donor APCs in recipient serum prior to transfusion. We have shown, however, that such incubation leads also to an increased expression of costimulatory activity and such cells are then immunogenic even following fixation [19]. To be fully effective in the inductions of donor specific tolerance, the incubation in recipient serum must be associated with some additional process that will inhibit the expression of costimulation.

An alternative approach is to interfere with the delivery of the costimulatory signal after it has been expressed on the membrane of APCs. Preliminary evidence suggests that the expression on APCs of cell adhesion molecules such as ICAM-1, ICAM-2 as well as the secretion of lymphokines such as IL-1 and IL-6 may be involved in costimulation and experimentation with monoclonal antibodies against these structures will provide a better understanding of these complex phenomena [19].

Understanding the mechanisms of allorecognition and proliferation of T cells into effector cells and, particularly, the means by which responding T cells can be diverted into anergy and allograft tolerance is of major importance in transplantation immunology. The prevention of graft rejection by the induction of donor specific tolerance without compromising the normal immune responses of the recipient is the only acceptable solution. We anticipate the time when graft rejection will no longer be the major obstacle to allografting and any tissue or organ can be a candidate for transplantation.

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DISCUSSION

H.T. Meryman, C.Th. Smit Sibinga

J.C. Bakker: I would like to congratulate Dr. Fahy with the important progress he has made over the last three years. I have a question: You talk about 100% viability. How did you define viability. Was it just transplantation or did you use an *in vitro* assay?

G.M. Fahy: It was a permanent transplant model. The treated kidney was put into the rabbit and the contralateral kidney was removed at the same time. The 100% viability reverses the fact that all the kidneys that you treated in that way will support life and not only that – the serum creatinine level will return to normal values within about 7 days or so. In the long term follow-up you see normal histology in all parts of the kidney. So, it is viability in terms of the post-transplant life support function of the kidney.

Ch. Körber (Aachen, FRG): Dr. Fahy, if I understood correctly, you mentioned that the effect of pressure is counteracted by the structuring of water due to the cryo-additives. Is this effect reflected then in a smaller depression of the liquidus curve than to be expected from the phase diagram, or is there other evidence that you can present in order to substantiate that statement?

G.M. Fahy: The statement is based on research done by MacFarlane and Forsyth.[1,2] One of the things that they did was to look at the volume changes associated with the crystallization of the medium, finding that "structure-making" solutes reduced or did not affect V, whereas electrolytes increased V. The answer to the question is no, you do not see an effect on the liquidus line as result of the structuring. In fact, you see what you know you see, which is that these solutions are non-ideal solutions and you get more of a depression of Tm than you would expect from ideal behaviour. The structuring is a second order effect superimposed

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onto that. The effect of structuring has so far only been visible at high pressures. The arguments so far as to how structure-makers counteract the effects of pressure on T_h are confusing and unclear, but the fact that they have this effect is clear. The primary evidence is that you see this counteraction only with known so-called kosmotropic agents. They organize water structure and that is thought to be the reason that they are kind to proteins and membranes and are non-denaturants. If you do pressurisation experiments on electrolytes, which are "structure-breakers", you find that the effects of the pressure are not counteracted by the solute and so presumably it is the structuring effect of the cryoprotectants that is responsible for the counteraction of the pressure effect. In fact, the more highly structuring the solute is, the stronger is its ability to counteract the effect of pressure.

L. Rydberg (Göteborg, S): Dr. Meryman, you showed us very nicely how T cells are activated by protein antigens, but do you know anything about carbohydrate antigens and the immune response to them. Are T cells at all involved and if so are the carbohydrate antigens handled like the protein structure?

H.T. Meryman: No, I do not think I can be too helpful there. Of course, many carbohydrates can be recognized directly by B cells without the necessity for antigen presenting cells or antigen processing. I am afraid I cannot give you an authoritative answer to what extent carbohydrates would be processed in the same form as protein.

J.N. Kearney: Dr. Novakovitch, did you assess the cellular viability of these bone products after freezing and were the bone cells actually living? How did you get the cryoprotectant to permeate the very dense cortical bone matrix?

G. Novakovitch (Marseille, F): About the cryoprotectant penetration, there is an incubation at 4°C for the long bones during 30 minutes before freezing. We have allografted since 1983 and patients now do walk without difficulties. That is our clinical answer.

J.N. Kearney: I am interested to know to what extent this bone was a living graft, as opposed to a dead implant. Because dead bone will become incorporated.

G. Novakovitch: The first aim is to stabilize, additionally we have looked at repopulation of the bone by the recipient through HLA typing. Two years after bone graft a young man had an accident and we could see the cells from the bone broken. We identified that the cells were the recipient cells.

C.Th. Smit Sibinga: Apparently there is an ingrowth also from the recipient and the bone is viable and does not serve only as a support.

J.N. Kearney: Right, because there was no expression of class I or class II HLA antigens and that could be explicable if the bone was actually dead when it was implanted.

H.T. Meryman: Also, as our experiments have demonstrated, the presence of class I and class II antigens in the absence of a viable antigen presenting cell will not induce an immune response.

C.Th. Smit Sibinga: Dr. Novakovitch, a question you raised yourself is could we deal without cryoprotectant. But the bone as you get it from the donor, not only has the bone per se but it has also a lot of supportive tissue around like vessels and stroma cells. Would you think that not using a cryoprotectant would affect these structures and that that might ultimately have an effect on the outcome in the viability, because of the need for some stroma for ingrowth of vessels from the recipient.

G. Novakovitch: Presently, I do not know. But vessel integrity is in our consideration permitting repopulation for the recipient cells. We have started a randomized study to answer this.

C.Th. Smit Sibinga: Dr. Mulder, it is intriguing that there was a definite difference between the fresh allogeneic platelets in the increment in the patients as compared to the autologous platelets. Yet, you observed that the intervals needed for the support were absolutely not different. Was the same true for the intensity of the bleeding tendency shown in these patients.

P.O.M. Mulder-Dijkstra (Groningen, NL): It is true that the increment of the allogeneic fresh single donor platelets was twice as much as in the cryopreserved autologous platelets. Our prophylactic treatment starts when the platelet count is below 15×10^9 platelets/litre and there was no difference between groups treated. Both groups had the same baseline platelet level and I cannot say anything about bleeding because there was no bleeding in both groups.

C.Th. Smit Sibinga: That apparently meant that the haemostatic capacity at least in the prevention of bleeding episodes was equal to those of fresh platelets.

P.O.M. Mulder-Dijkstra: Yes, it certainly was.

A. Brand: Dr. Meryman, you presented the role of the APC's in the induction of immunization and tolerance. If you had to make a decision

for blood transfusion, not for organ transplantation, either to remove the APC's, so that immunization would not take place or to treat the blood with UV light or chemical substances to inactivate the APC's, and to induce a kind of tolerance, what at this moment – if you look to the two procedures to prevent immunization from blood transfusion – would you prefer in the light of the experiments you have done.

H.T. Meryman: I think, without question, that depleting the blood product of lymphocytes is preferable. If you use a chemical substance to inactivate, then you are going to have to worry about infusing that material into the recipient or washing the cells to get rid of it. There has been an accumulation of data, particularly from Europe, on the use of leukocyte-depleted blood products. We also have a study in collaboration with Johns Hopkins hospital. It looks as though reducing the total number of lymphocytes per transfusion to 5×10^6 or fewer is going to be sufficient to prevent alloimmunization. This is a level that is achievable by the latest generation of filters. I feel that in situations where patients are going to receive multiple transfusions over a period of time, where there is an opportunity for alloimmunization and where this is undesirable, both red cells and platelets should be filtered. I think this is particularly important in the dialysis centres. Back in the early 70's, dialysis centres were using frozen red cells because they were poor in leukocytes and it was correctly expected that that would reduce alloimmunization, which was at that time thought to have an adverse effect on kidney transplants. In the United States roughly 100,000 units of frozen red cells each year were used in dialysis centres. When Opelz and Terassaki[1] reported that transfusions improved kidney transplants, frozen red cells were abondoned and packed red cells were used. Now we find that this transfusion effect is vanishing. The use of cyclosporin and better management of patients appear to have eliminated the advantages resulting from transfusions prior to transplantation. Now we need to have concern for those patients in the dialysis centres, who are being alloimmunized and never get a transplant because they are so broadly alloimmunized. That is a place for the mandatory use of filtered red cells. I expect to see the use of filtered cells expand substantially, but only appropriately in those cases where alloimmunization would be expected to be a problem.

A. Brand: So you do not advise to invest all the money and energy in UV irradiation of blood.

H.T. Meryman: I have some misgivings about the UV irradiation of blood, because we know that UV-irradiated APC's will induce some degree of tolerance. Now, is immunosuppression a good thing for a patient who

1) Opelz G, Terassaki PI. Transplant Proc 1977;9:121-2.

does not need immunosuppression? As you may know there are some indications from a series of excellent studies by Tarttar[1] that there is an increased incidence of infection in surgical patients who have been transfused. One could obviously draw the conclusion that the immunosuppression from transfusion was increasing the risk of infection. I do not know whether that is going to be confirmed, but UV irradiation is known to induce a degree of immunosuppression beyond that expected from just the conventional transfusion. This makes me a little nervous about UV.

C.Th. Smit Sibinga: Dr. Novakovitch, another question. You showed a marvellous result on both the bone in small particulate form in the jaws as well as in the larger structural transplants. Do you have any experience with cartilage.

G. Novakovitch: No, I have not.

C.Th. Smit Sibinga: Has it ever occurred to your opinion that a transplant in a child still in the growing age has been done with a bone transplant which included the growth nuclei, such that the maturation lengthening of the bone could futher proceed.

G. Novakovitch: We have done bone grafts for tumours like Ewing's tumour and femoral tumour for the children. But I cannot demonstrate this.

C.Th. Smit Sibinga: It would be marvellous if that really could work, because then in certain situations in children one might have the opportunity for further maturation of the bone and lengthening.

1) Tarttar PI. Transfusion 1989;29:456-9.

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