The modern history of blood transfusion began with Landsteiner's classic description of the A, B, and O blood groups. Specific typing and crossmatching techniques can now be applied with all erythrocyte antigens and have virtually eliminated major transfusion reactions due to red blood cell incompatibility.

Practical application of Landsteiner's discoveries awaited the development of a suitable anticoagulant to permit blood to be stored for short periods between collection and transfusion. Hustin, in 1914, showed that trisodium citrate could be used for this purpose. Subsequent progress in transfusion therapy has resulted from parallel advances in the fields of blood group immunology and blood preservation.

Rous and Turner, in 1916, showed that the addition of glucose to citrate improved results over those with citrate alone. Their modification of the original anticoagulant solution made it possible to establish blood banks in France during World War I. Although Rous-Turner solution was an excellent preservative, the large volumes of solutions and the necessity to autoclave the glucose and citrate separately, lead to disuse of this method of blood preservation.

In 1943, Loutit and Mollison found that glucose and citrate could be autoclaved together if the solution was acidified with citric acid. Acid-citrate-dextrose anticoagulant is now in use throughout the world and permits whole blood to be refrigerated at 4°C. Approximately 70% of the cells remain viable for 21 days following collection. Widespread use of ACD solution for collection and storage of blood has changed the entire scope of modern medicine. Developments in cardiac, vascular and radical cancer surgery over the past ten years would not have been possible without blood replacement therapy.

Routine use of blood transfusions and their relative freedom from major erythrocyte incompatibility reactions have lead to progressively increased demands for blood. At the Massachusetts General Hospital Blood Bank, 50% of the blood is dispensed to patients who receive more than 5 liters. Transfusion of sick patients with large volumes of ACD blood frequently leads to acidosis, impaired cardiac function and bleeding. Although liquid blood has proved to be very useful, it also has major limitations. Febrile reactions due to leukocyte and platelet sensitivity are being seen with increasing frequency in recipients of multiple transfusions. Graft versus host reactions in which lymphocytes of fresh blood form antibodies against the recipient and lead to hemolytic anemia have been identified following open heart surgery. Communicable disease—e.g. syphilis, malaria, hepatitis, and possibly leukemia—can be transmitted by
Current efforts to prolong the useful storage of red blood cells by the use of citrate-phosphate-dextrose (CPD) anticoagulant or by the addition of adenine nucleotides to ACD-collected blood may help to improve the viability of the erythrocytes\(^7,8\). It is unlikely, however, that either of these methods will have significant effect on the metabolic or donor-recipient problems inherent in massive whole blood transfusion. "Liquid storage methods have described by Dr. John Gibson as delayed deterioration" rather than preservation\(^7\).

An ideal technique for blood preservation would employ cryogenic temperatures to arrest the aging of erythrocytes and cell washing to purify whole blood and reduce the number of its extraneous components. Neither measure by itself would be as beneficial as the two combined.

Initial experiments in our laboratory were designed to study the events that take place during the slow freezing and thawing of human blood. When water freezes, intermolecular hydrogen bonding between water molecules leads to ice crystal formation. Ordinary solutes do not participate in this type of electrostatic bonding, are excluded from the water-lattice structure, and become concentrated in the small percentage of liquid water that remains in the interstices between the crystals of pure water. Concentration of solutes by freezing is illustrated diagramatically in Figure 1.

Lovelock, in 1953, found that human erythrocytes are damaged by contact with concentrated sodium chloride solutions\(^9\). Our studies have confirmed Lovelock's findings and have shown that both human red blood cells and platelets are damaged whenever sodium chloride concentrations exceed 0.8M. Miura has recently found that canine erythrocytes and whole perfused canine kidneys are also damaged irreversibly by salt solutions stronger than 0.6M NaCl\(^10\).

The solute exclusion effect of freezing can damage living cells in a variety of ways\(^11\). Concentrated solutes alone can be injurious, as illustrated in the experiments with human and canine tissues. The pH of biological fluids can vary greatly as the result of changes in the ratio of concentrations of salts in buffer systems. Water of hydration binding lipoprotein units together can be removed causing denatur-

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**FIG. 1: Solute exclusion during freezing. The water in ice is pure. The dissolved solutes become concentrated.**

[Diagram of liquid saline and frozen saline with solute exclusion illustrated.]
ation. It is now clear that the solute exclusion during slow freezing exerts a general damaging action. Excessive concentrations of solutes may not be the sole cause of cellular death during slow freezing and thawing, but they are an adequate cause of injury and must be avoided if blood or other tissues are to be preserved in the frozen state 12).

Polge, Smith and Parkes found a solution to many of the problems faced by living cells at low temperatures when they discovered the remarkable ability of glycerol to protect fowl spermatozoa against death during slow freezing and thawing 13). Dr. Audrey Smith later reported that glycerol had to be removed from the thawed cells to prevent osmotic hemolysis when the erythrocytes were resuspended in saline 14).

Many methods have been developed to freeze blood in the presence of glycerol and satisfactory preservation of erythrocytes has resulted 15). Practical considerations of cost, Processing time, cumbersome techniques, and bacterial sterility, however, have kept these methods from achieving widespread clinical usefulness.

In 1959, Lovelock and Bishop, reported that dimethylsulfoxide (DMSO) protected living cells against freezing injury in a fashion similar to glycerol 16). The compound crossed cellular membranes rapidly and protected the glycerol-impermeable erythrocytes of cattle. We used dimethylsulfoxide in all of our early studies and found it to afford virtually complete protection to large volumes of human blood against hemolysis during slow freezing and thawing 17). Although blood preserved with this additive gave excellent clinical results, uncertainty regarding toxicity led us to search for methods by which the normal body constituent, glycerol, could be substituted for dimethylsulfoxide.

Dimethylsulfoxide and glycerol are members of a class of chemical compounds-endocellular cryoprotective agents (ECA). All strong ECA's share the property of forming very firm hydrogen or coordinate bonds to water molecules. The presence of an ECA bound tightly to water molecules interferes with their orderly deposition into ice, reduces the percentage of cellular water sequestered in ice, and thereby minimizes the degree to which dissolved solutes concentrate in the frozen state. This concept is illustrated diagramatically in Figure 2. The degree to which the solutes concentrate is reduced in logarithmic proportion to the concentration of the ECA 17). The cryoprotective action of glycerol and dimethylsulfoxide is identical when these compounds are present in blood in equimolar concentrations. A 5.0 to 5.7 M cellular concentration of either glycerol or dimethylsulfoxide permits treated human erythrocytes to be frozen and thawed slowly with minimal hemolysis. The use of ECA's in high concentrations assures positive control against solute excess and provides a safeguard against cellular damage secondary to minor variations in storage temperature. ECA-treated cells can be thawed and refrozen if desired.

Slow freezing is desirable in any method for preserving blood because it avoids cellular damage from ice formation inside the blood cells. The injurious nature of intracellular ice crystals has been demonstrated elegantly by Prof. Asahina and Prof. Nei of the Institute of Low Temperature Science of the University of Hokkaido 18,19). Slow freezing is particularly desirable from a practical standpoint because the technical requirements for refrigeration, packaging, and processing of blood are not critical.

ECA's diffuse into erythrocytes without concentration gradient, and exert no net osmotic action upon living cells. The action of ECA's and distilled water are similar in this regard. An external solute must be added to ECA solutions to maintain osmotic equilibrium. If the added solute is hypertonic, the
percentage of hemolysis after storage for 24 hours at \(-85^\circ\)C. is greater than that found with similar cells frozen in isotonic media \(20\). Hemolysis in hyper- or isotonic media, however, does not increase with prolongation of storage in the frozen state. If the ECA solution is hypotonic, however, hemolysis in blood samples thawed after 24 hours is minimal but increases with storage at \(-85^\circ\)C. The rate of progression of hemolysis during storage is related to the degree of cellular swelling before freezing and is maximum for cells just smaller than their critical bursting volume. The destruction of swollen erythrocytes that takes place during storage at low temperatures occurs whether NaCl, sucrose, or glucose is the solute and appears to be an osmotic rather than a specific chemical function of the additive. Hypotonic hemolysis of swollen erythrocytes during storage can explain the wide variation in reported results of long-term storage of erythrocytes in glycerol at low temperatures. The degree of success of human erythrocyte preservation is determined both by the concentration of the ECA and that of the external solute in the prefreezing medium \(17\).

Dr. Audrey Smith found that ECA’s must be removed from thawed blood to prevent osmotic hemolysis on transfusion. Techniques that have proved effective for removal of ECA’s include dilution, dialysis and serial or continuous centrifugal washing. The ECA is removed by its dilution in a large aqueous phase. Physical-chemical methods designed to separate ECA’s from water (e.g. partition, precipitation or fractional distillation techniques) have all been tried and found to be ineffective.

In the search for new ways to remove ECA’s from thawed glycerolized blood, we studied the clumping that occurs when blood is diluted with sugar solutions. This phenomenon is seen in an intravenous tubing set whenever a blood transfusion is followed by dextrose in water.

Our studies led us to conclude that \(\gamma\)-globulins and red blood cells normally exist together as separate
entities in plasma (Figure 3). In the pH range 5.2 to 6.1, however, a reversible complex is formed between γ-globulins of the plasma and lipoproteins of the red blood cell wall (Figure 4). If the ionic strength of the slightly acid medium is lowered, the globulins precipitate with co-precipitation of the attached erythrocytes (Figure 5). It is a fortunate coincidence that the pH of ACD blood and autoclaved sugar solutions are optimum to promote agglomeration. Agglomerated cells may be resuspended either by the addition of electrolyte, breaking the γ-globulin-γ-globulin bond, or by raising the pH, breaking the γ-globulin-lipoprotein bond (Figure 6). The phenomenon has been called reversible agglomeration.
REVERSIBLE AGGLOMERATION OF ERYTHROCYTES

\[ \text{pH} = 5.9 \]
\[ \text{I/2} = \text{Low} \]

\[ \gamma G \rightarrow \gamma G \]

\[ \text{L-P} \]

\[ \text{L-P} \]

Fig. 5: When the ionic strength is reduced the \( \gamma \)-globulins precipitate with co-precipitation of the erythrocytes. The cells agglomerate.

RESUSPENSION OF AGGLOMERATED CELLS

\[ \text{I/2} \]

\[ \text{pH} \]

\[ \gamma G \]

\[ \gamma G \]

\[ \text{L-P} \]

\[ \text{L-P} \]

Fig. 6: Agglomerated cells may be resuspended either by addition of electrolyte, or by raising the pH.

to distinguish it from agglutination and aggregation, both of which have specific hematologic connotations. Although the mechanism of action of reversible agglomeration may prove to be more complex than our hypothesis would suggest, it does, however, explain observed facts and has been found useful.

The foregoing biological principles of cell preservation and cell washing have been combined to form
the scientific basis of a 3-phase system for long-term blood preservation. In the first phase a solution of an endocellular cryoprotective agent (glycerol) containing nonelectrolyte solutes (glucose and fructose) is added to packed ACD blood to protect the cells during freezing, storage and thawing. In the second phase the ECA is removed from the thawed blood by its dilution in aqueous solutions of sugars. Reversible agglomeration of erythrocytes serves to re-concentrate the cells after each of three serial 8:1 dilutions and eliminates the need for centrifugation at this stage. In the final phase the sugar-washed agglomerated cells are resuspended by the addition of any solution that contains electrolyte or raises the pH of the medium above 6.7. This prepares the washed cells for transfusion.

All processing steps before freezing and after thawing can be accomplished simply, rapidly and effectively using the International Equipment Company Cytoglomerator* and its disposable plastic Blood Freezing Units. The frozen blood may be stored conveniently in a standard 16 ft.® Harris —85°C. twin dual cascade electrical deepfreeze**. Fifteen units of blood can be thawed and washed each hour using this equipment.

**TECHNIQUE

In practice, packed cells from blood collected in Acid-Citrate-Dextrose are transferred via a sterile

*Manufactured by International Equipment Company, Needham Heights, Massachusetts. Huggins Cytoglomerator Model WS #3550; Blood Freezing Unit #3575; Diluent-reconstitution sets #3574.

**Manufactured by the Harris Manufacturing Company, Cambridge, Massachusetts Model 16L-2-D10.
spike coupler from the donor's collection container to the lower end of the Blood Freezing Unit on the Cytoglomerator (Figure 7). A volume of autoclaved 8.6 M glycerol is added to an approximately equal volume of the packed cells. Each liter of the 8.6 M glycerol solution is made to contain 80 Gm. of glucose, 10 Gm. fructose and 3 Gm. of sodium ethylenediaminetetraacetic acid (EDTA). The sugars in the 8.6 M glycerol solution act to maintain physiologic isotonicity. The EDTA chelates calcium and prevents the irreversible binding of β-globulin to the erythrocytes that occurs in non-electrolyte solutions 23). The EDTA thereby eliminates the possibility of falsely positive Coombs tests. After addition of the glycerol the Blood Freezing Unit is folded, replaced in its box, and placed to freeze in the deep-freeze at −85°C.

After storage at low temperature the Blood Freezing Unit and its contents are thawed in the water-bath (+40°C) and again attached to one of the five operating stations of the Cytoglomerator. The thawed cells are washed by sequential addition of 500 ml. of 50% glucose and the contents of two 3000 ml. bottles of 8% glucose and 1% fructose. After the first 2500 ml. of sugar solution has been added the agglomerated cells are allowed to settle and the supernatant is decanted. Dilution and decantation of excess wash solution is repeated twice.

In this study the cells were reconcentrated by centrifugation, the supernatant was removed and the blood was administered to patients as packed cells. Although bacterial cultures on blood agar, thioglycollate and Sabouraud's media have been consistently negative, the resuspended cells were generally used clinically within 24 hours of washing.

RESULTS

To date (10 March 1965) 651 full clinical units of blood have been frozen in the presence of ECA's stored at −85°C, for periods up to one year, and administered clinically to 93 patients—an average of 7 units per patient. Dimethylsulfoxide was the ECA used for the first 133 units, propylene glycol for three, and glycerol for the most recent 515.

The percentage of hemolysis of erythrocytes at different stages of processing is shown in Table I. When frozen cells are administered as packed cells the recipient receives between 10 and 70 mg. of free hemoglobin per unit. The quantity is less than that frequently found in 21-day-old ACD blood.

Recovery of erythrocytes through in vitro processing averaged 90±5 per cent. Radioactive chromium survival studies showed 87 to 95 per cent of the labelled erythrocytes to be present in the recipient's circulation 24 hours after transfusion with a normal slope of decline of radioactivity thereafter (T1/2 = 30.5 days). These cell survival results have recently been confirmed independently by Valeri, et al. at the U.S. Naval Hospital, Chelsea 24). No detectable deterioration has been seen with cells stored for periods longer than one year at −85°C. The 80 per cent over-all efficiency of the process (% in vitro recovery × % in vitro survival ÷ 100) compares favorably with the 70 per cent 24 hour survival of cells

<table>
<thead>
<tr>
<th>TABLE I HEMOLYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent Samples = 1.36% σ = 1.00%</td>
</tr>
<tr>
<td>Full Units = 1.27% σ = 0.77%</td>
</tr>
<tr>
<td>Final Blood = 0.29% σ = 0.23%</td>
</tr>
</tbody>
</table>
in ACD blood stored for 21 days at 4°C.

The biochemical status of the resuspended erythrocytes is shown in Table II. Of particular clinical importance is the low level of potassium and virtual elimination of protein, blood group isoagglutinins, and viable white blood cells.

**TABLE II FROZEN BLOOD (Group O)**
(Resuspended with 250 ml. 0.85% NaCl)

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium</td>
<td>84.3 ± 11.6 meq/l</td>
</tr>
<tr>
<td>Chloride</td>
<td>85.2 ± 9.3 meq/l</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.9 ± 0.62 meq/l</td>
</tr>
<tr>
<td>Glucose</td>
<td>4.5 ± 0.6 Gm.%</td>
</tr>
<tr>
<td>Citrate</td>
<td>0</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0</td>
</tr>
<tr>
<td>WBC</td>
<td>650/mm³</td>
</tr>
<tr>
<td>Anti-A titer</td>
<td>nil - 1:4 [both saline and immune]</td>
</tr>
<tr>
<td>Anti-B titer</td>
<td>nil - 1:4</td>
</tr>
</tbody>
</table>

Averages and standard deviation: 50 consecutive units.

Clinical studies have shown consistent uneventful sustained elevation of hemoglobin and hematocrit in anemic recipients. The *sine qua non* of effectiveness and safety of any blood preservation technique has been fulfilled.

**DISCUSSION**

Temperatures in the range of −85°C. effectively arrest the aging of human erythrocytes and permit red blood cells to be stored for long periods. We have used blood stored for longer than 18 months and expect that it should last for at least five years. Washing removes unnecessary and potentially harmful products and makes frozen blood of particular advantage in selected clinical situations. The ability to thaw and wash multiple units of frozen blood in the same amount of time required for crossmatching makes the system applicable to general clinical use.

The removal of both saline and immune Anti-A and Anti-B blood group isoagglutinins during the washing phase eliminates the potential hazard of group O blood as "universal donor O". We now freeze group O blood exclusively for use in recipients of any ABO blood group. This practice has given good clinical results and simplifies the practical problem of blood storage.

Dr. Morten Grove-Rasmussen, Director of the Massachusetts General Hospital Blood Bank and Transfusion Service, has recently completed a statistical study of hemolytic reactions that occurred during transfusion of 1.5 million units of blood [25]. Antibodies within the Rh system [anti-D, C(CD), E and c], Anti-Kell (K) and Anti-Duffy (Fya) had the greatest clinical significance. His finding led him to suggest that we freeze two specific types of Group O blood:

1) Group O, Rh-neg. (DCE-neg.), K-neg., Fya-neg. can be used for a recipient of any ABO group sensitized to the irregular antigens, D, C(CD), E, K, or Fya.

2) Group O, Rh-pos. (R R) (DCE/DCE), K-neg., Fya-neg. can be used for a recipient of any ABO group sensitized to any of the irregular antigens, c, E, K, or Fya.

These two types of Group O blood freed of their Anti-A and Anti-B isoagglutinins will eliminate the possibility of hemolytic transfusion due to ABO incompatibility and 80–90% of hemolytic reactions due
to irregular blood group antibodies. Donors with these two special blood types are found in 4 to 5% of the random caucasian donor population. An example of the inventory of the different types of blood that we maintain in the frozen state is shown in Table III. Blood of rare types is preserved as a part of the Reference Laboratory Program of the American Association of Blood Banks 26).

**Table III FROZEN BLOOD INVENTORY**  
(5 March 1965)

**COMMON:**
- RhD positive 35
- Rh CDE negative 30

**UNCOMMON “Universal”**
- CD\textsubscript{e}CD\textsubscript{e} K\textsuperscript{-} Fy\textsuperscript{a}\textsuperscript{-} 45
- cde/cde K\textsuperscript{-} Fy\textsuperscript{a}\textsuperscript{-} 52

(All routinely typed for MN, P, JKa, Le\textsuperscript{a+b})

**RARE:**
- RhD pos. KK (cellano neg.) 3
- RhD pos. Lu\textsuperscript{a} Lu\textsuperscript{a} (Lu\textsuperscript{b} neg.) 5
- RhD pos. Lw\textsuperscript{-} neg. 1
- cde/cde (rr) Vel-neg. 1
- CD\textsubscript{e}/CD\textsubscript{e} (R1\textsuperscript{c}) Vel-neg. 1

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Although the erythrocyte antigens in the Japanese population have different specificity than those listed above, the basic approach to the clinical problems of blood group antibodies could be used. To do this requires a knowledge of the statistical frequency of antibodies responsible for hemolytic reactions and the incidence of blood group genotypes in the random donor population. The concept of specific donor selection by statistical usefulness may modify the generally accepted concept of random collection for random need.

During the course of washing after thawing the anticoagulant, excess potassium and other electrolytes are removed. This purification of whole blood has proved useful in the treatment of uremic and hypocalcemic patients. No foreign protein or urticarial reactions have been encountered even in sensitized recipients.

Removal of the donors plasma suggests that the absolute amount of serum hepatitis virus carried in frozen blood might also be reduced. Clinical cases of all infectious diseases are the product of the dose of infectious agent and the resistance of the host. Reduction of the dose of virus by washing might, therefore, be reflected by a lesser incidence of serum hepatitis following transfusion with frozen blood than is seen at present with ACD blood.

No cases of homologous serum hepatitis have been encountered following administration of blood frozen by our system. The clinical series, however, is small and the attack rate of hepatitis in the Boston area is low. “Absence of cases of serum hepatitis, therefore, does not necessarily indicate a casual relationship.” In any event a person anticipating surgery could have his own blood frozen for autotransfusion when needed. Autotransfusion is the only certain method for preventing homologous serum hepatitis.

White blood cells and platelets are removed during preservation of blood by freezing. The leukocytes are killed during slow freezing and thawing in the 5.0 to 5.6M glycerol. The optimum glycerol
concentration for leukocyte preservation is 1.9 to 2.2M. The debris of dead white cells and platelets is removed during the washing phase after thawing.

Blood without viable white blood cells has major clinical usefulness in patients with leukoagglutinins. In our experience, this type of patient can be given frozen blood without developing febrile reactions.

Elimination of viable histocompatibility antigens makes frozen blood of particular value for kidney homotransplant recipients, and potential recipients. We now use frozen blood routinely in this group of desperately ill patients.

At the present time frozen blood accounts for 4% of the output of the Massachusetts General Hospital Blood Bank.

**SUMMARY**

A method has been developed to preserve blood at cryogenic temperatures. The technique is simple, relatively inexpensive, and rests on a firm physiologic basis.

In addition to the long-term storage characteristics, the resuspended frozen blood has attributes that make it superior to ACD collected whole blood in selected clinical situations. The concept of specific donor selection by statistical usefulness may modify the general concept of random collection for random need.

**REFERENCES**

6) Grove-Rasmussen, M.: Personal communication.
17) Huggins, C.E.: Prevention of hemolysis of large volumes of red blood cells slowly frozen and thawed in


24) Valeri, C.R.: Personal communication.


Dr. Huggins の講演に対して

国立東京第1病院 島 居 有人

血液の凍結保存については、ただ今の講演にあったGlycerol添加の方法が主流であるが、他に液体窒素中に血液をつぎ込む方法とかglucoseおよびLactose を加えて急速冷凍をする方法とかがある。Glycerol添加法で一番問題となるのは融解した血球からGlycerolを洗い出すことであるが、CohnのFractionatorなど考察されたが、煩雑で時間もかり実用的でなかった。このたびDr. Hugginsは糖液添加による迅速洗浄法を用いて遠心沈澱の必要をなくし、臨床的にも使い易くなった点は大変すくされている研究であると思う。なお将来は全血を、そのまま短期凍結保存ができるよう研究が発展することをのぞんでいる。

Dr. Huggins の講演について

北大低温科学研究所 梶井 外喜男

ただ今、お聞きの通りDr. Hugginsは、血液の凍結の問題について、これまでに達しなかった多くの人々の研究を要領よくまとめて紹介し、われわれの理解を深めてくださり、またDr. Huggins自身、いろいろと立派な業績をあげておられますが、今日のお話しにもありましたように、ブドウ糖を用いての血球の沈澱分離や凍結保存用のプラスチックの袋の考案などは、そのうちでも特色のあるものといえましょう。さて、こうしたお話をうかがうにうつげて感じますことは、わが国では、この方面の研究発表が殆どないということです。

1960年に東京で開催の国際輸血会議にも出席された20分野の専門家の1人、Dr. Rinfretが、昨年New Yorkでの低温生物学会の折に、私につきのようなことを申しました。「東京の後、Mexico City, Stockholmと、相ついての国際輸血会議に日本から多数の学者が出席しておられたが、血液の凍結の問題に関しては一題も発表がなかったのは大変残念に思う」とのことでした。

Dr. Hugginsの来朝を機会に、この分野の研究に関心をもたれる方のご発言を期待してやみません。