FROZEN BLOOD

A Review of the Literature 1949–1968

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FOREWORD

The Biological Sciences Communication Project (BSCP), in conducting studies of the literature on various aspects of blood preservation for the U.S. Navy, Bureau of Medicine and Surgery, and Office of Naval Research, became aware of increasing scientific attention being focused on the preservation and storage of blood in the frozen state. Since 1949, when it was discovered that glycerol would protect red blood cells against damage by freezing and thawing, there have been significant developments in the processing and uses of frozen blood. Since a wide variety of concepts is embraced in the subject matter dealing with one facet or another of frozen blood, it appeared to us at the BSCP that it would be valuable to review the literature on frozen blood as a specialized segment of the study of the literature on blood preservation.

Collecting the material needed for this review proved to be a task far more formidable than had been anticipated. In addition, the review as originally conceived focused on the erythrocyte, and it was not until work had been in progress for some time that the inclusion of other formed elements of the blood was suggested. As a consequence, in this respect, the study cannot pretend to be all-inclusive, and some important references may have been missed. However, it is hoped that readers will find the presentation useful.

Charles W. Shilling, M.D.
Director, BSCP

December, 1969

PREFACE

This review of the literature on the cryopreservation of blood encompasses the period of 1949 through 1968. In general, although reference is made (particularly in the chapter on History) to publications dated prior to 1949, they are cited in the text but are not included in the bibliography. The citations included in the bibliography are largely limited to those that deal with frozen blood. Papers concerned with refrigerated, but not frozen, blood have generally been excluded. During the preparation of this review, several instances were found of a paper having been presented at an international meeting which was antedated by the publication of an abstract. In such cases, the bibliographic citation is that of the full article with a notation that it appeared in abstract form, for which the appropriate citation is given. Occasionally the same article, or at least the same material on which the article was based, has been published as many as four times in different publications at different times in the same year, or in different years as much as two years apart. In such cases these are combined into one bibliographic citation.

Numerous articles reviewed for this study exist in abstract form only. For the most part, they were found in reports of the *Proceedings* of an international meeting or a similar gathering such as a symposium or a conference. A few articles which should have been included in this review came to our attention too late, or could not be obtained from the library until after the suspense date. Some were at the bindery, for example, or not in their proper places on library shelves. Therefore, a certain number of references published during the time period under review have not been included. A selected list of abbreviations is intended to define and clarify some of the symbols and specialized terminology commonly used by investigators in this field.

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trade names or commercial products does not constitute or imply recommendation or endorsement of such products by the contributors or editors of this volume, or by, or on behalf of, any branch of The George Washington University or the United States Navy.

Arthur R. Turner

December, 1969
Washington, D.C.

ABBREVIATIONS

ACD	Acid-citrate-dextrose solution, an anticoagulant
ACD-A	U.S.P. formula A of ACD solution
ADL	Arthur D. Little Co., manufacturer of Cohn fractionator
ADP	Adenosine diphosphate, a nucleotide
AMP	Adenosine monophosphate, a nucleotide
ATP	Adenosine triphosphate, a nucleotide
CPD	Citrate-phosphate-dextrose solution, an anticoagulant
DMSO	Dimethyl sulfoxide, a cryoprotective agent
DPG	Diphosphoglycerate
DPN	Diphosphopyridine nucleotide, a coenzyme, the dinucleotide of nicotinamide and of adenine
ECA	Endocellular cryoprotective agent
EDTA	Ethylenediaminetetraacetic acid, an anticoagulant
EOP	Efficiency of process
GDH	Glutamic dehydrogenase
GOT	Glutamic oxaloacetic transaminase, an enzyme
GPD	Glyceraldehyde phosphate dehydrogenase, an enzyme
G-6-PD	Glucose 6-phosphate dehydrogenase, an enzyme
GTP	Guanosine triphosphate
HDP	Hexose diphosphate
HES	Hydroxyethyl starch, a cryoprotective agent
HMP	Hexose monophosphate
5-HT	5-hydroxytryptamine, or serotonin
IMP	Inosine monophosphate
LDH	Lactate dehydrogenase, an enzyme
M	Mole
mM	Millimole
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
PVP	Polyvinlypyrrolidone, a cryoprotective agent
PVP-K17	A PVP with molecular weight of 10,000 to 20,000
PVP-K30	A PVP with molecular weight of about 40,000
SH	Sulfhydryl enzyme
TPD	Triose phosphate dehydrogenase
TPN	Triphosphopyridine nucleotide

CONTENTS

Chapter 1

HISTORY

Sources of Historical Summaries

Much of the work up to 1935 on the action of low temperatures on living organisms has been summarized, according to Smith (1954), in a monograph by Belehradek, J.: Temperature and Living Matter. *Protoplasma–Monographien* No. 8. Borntraeger, Berlin, 1935; and summarized up to 1940 by Luyet, B.J., and Gehenio, P.M.: Life and Death at Low Temperatures, Monograph published by *Biodynamica,* Normandy, Mo., 1940. (Reprinted from *Biodynamica* No. 38, 1938; No. 48, 1939; and No. 60, 1940.) Strumia, M.M. (1954) in a footnote on page 1106 called attention to the fact that practically the entire July 1947 number (Vol. XXVI, pages 591-755) of the *Journal of Clinical Investigation* was devoted to a series of articles on the basic principles underlying the in vitro storage of red blood cells and their posttransfusion survival, which he says gives a comprehensive view of much of the work on blood preservation done up to that time.

M.M. Strumia's article (1954) discussed major contributions to the solution of the problem of blood preservation from 1947 to 1953 and stated, "...in that period there was no practical and substantial contribution to the problem of longer storage of blood save for the promising although still highly experimental and impractical storage of erythrocytes in the frozen state in a glycerol solution." Smith (1954) covered a wide variety of subjects in the field of low-temperature biology "particularly in the light of work done between 1940 and 1952 on isolated cells and tissues of animal origin." The subjects treated include thermal shock, removal of water, velocity of temperature changes, effects of extracellular crystallization, intracellular freezing, cryoprotective substances, duration of survival at low temperatures, and effect on enzyme activity. Mollison (1954) reviewed advances in erythrocyte preservation. Jones, N.C.H., et al (1957) presented a review of previous work on frozen blood beginning with Smith (1950) and including Chaplin et al (1956).

Tullis (1964) gave a brief historical review of cryopreservation of erythrocytes and covered such subjects as the effects of freezing unprotected cells, methods of protection at subfreezing temperatures, physical changes during freezing, glycerol and DMSO additives, and nonpenetrative additives. MacFarlane (1964) provided a capsule summing-up of the utility of long-term preservation of blood. Doebbler (1966a) reviewed the majority of compounds or classes of compounds which have been used to provide protection against injury during freezing and thawing of such cell systems as tissue culture cells, mammalian bone marrow, spermatozoa, mammalian erythrocytes, vinegar eels, bacteria, plant cells, and human blood cells. Carlsson et al (1967) surveyed the development in Sweden, during the period 1949-1967, of various cryotechnical methods for the preservation of blood with special reference to its use in the military medical defense services. Glycerol and sugars in concentrated solutions have been used during, or prior to freezing to enhance resistance to low-temperature injury since 1913.

Luyet and Rapatz (Abst. 1967) revealed plans for preparation of a comprehensive bibliography and historical survey of studies on erythrocyte cryopreservation. Publication is scheduled in an issue of *Cryobiology* early in 1970. Hurn (1968) cited 470 references in his monograph, no part of which may be quoted without permission. However, it is stated on the dust jacket that "newer methods of frozen storage at very low temperatures are discussed in detail...Individual chapters are devoted to the special difficulties involved in the preservation of red cell antigens, platelets and other blood components including leucocytes." He has provided a synoptic overview of the blood-storage problem from the earliest experience in transfusion through the gamut of preservation methods, additives, and hazards. No aspect of the subject has escaped his attention: red cell lesions occurring in storage, freezing-thawing parameters, blood fractions, collection and storage of platelets, and utilization of erythrocytes in blood group tests are included.

Priorities

It has been noted by various authors since 1958 -- Steinbuch & Quentin (1958); Meryman (1959); Rinfret & Doebbler (1960a); Smith (1961); Bronson & McGinniss (1962); Rinfret (1963c); Richards et al

(1964); Perrault (1965) (who quotes the wrong reference); Doebbler et al (1966b); Carlsson et al (1967); and Merjanian (1968), to name a few -- either that Luyet was the first to report preservation of erythrocytes by very rapid freezing (Luyet, 1949, 1949a) or that modern cryobiology began with Luyet's work. It should be noted only in passing that Luyet's presentation at the conference in Boston on January 6, 1949, was not the only report on the freezing of blood (see Strumia, M.M., 1949, and Walter & Gibson, 1949). It is also to be noted that several authors (Smith, 1950; Sloviter, 1951a, 1953; Tullis et al, 1958; and Smith, 1961) have pointed out either that Luyet achieved success only with minute quantities of blood under very special conditions, or that because of those facts, his technique was mainly of academic interest, perhaps (as noted by Rinfret, 1965) because he did not suggest practical application of his observations. Meryman & Kafig (1955a), in reviewing Luyet's work (1949), stated, "...there are no other reports known to the authors in which the surface-to-volume limitations for rapid heat exchange were adequately recognized," and Tullis (1964) noted that Luyet had shown experimentally that the heat capacity of blood is clearly related to the thickness of the film being cooled.

Many authors (at least 40) cited the reports of Polge et al (1948) and Smith (1950) as providing the stimulus to renewed interest in cryopreservation of erythrocytes. Polge et al (1949) reported work dealing mainly with glycerol cryoprotection of fowl spermatozoa. Parkes (1951) cited the Polge et al (1949) report and stated, in part, "...it was discovered by a chance observation that glycerol had remarkable properties in protecting fowl spermatozoa against the effects of low temperatures." Smith (1961) cited the Polge et al (1949) report and stated, in part, "In 1949 new possibilities were opened up by the demonstration that glycerol protected avian spermatozoa against the otherwise damaging effects of freezing to, storage at, and thawing from very low temperatures. This led indirectly to resumed work on freezing red blood cells....It occurred to me that mammalian blood cells, which are excellent osmometers, could be used to test the osmotic effects of the semen diluents containing glycerol." She then went on to quote her own earlier report (Smith, 1950) and stated, in part, "When glycerol-treated red blood cells were frozen to and thawed from -20°, -40°, or -79°C. in the presence of 10 or 15 percent glycerol, the great majority of the cells were intact...:The majority of

erythrocytes were unaltered in appearance after storage for periods varying from 5 minutes to 3 months at -79°C. in plasma containing 15 percent glycerol." In light of this quoted material, it is indeed confusing to find the report of Polge et al (1949) cited in at least nine references as dealing with glycerol protection against freezing of spermatozoa from bulls.

The protective action of glycerol on fowl spermatozoa against damage caused by freezing was compared by Polge et al (1949) with that of other substances. It was found that propylene glycol, ethylene glycol, and other related substances were more toxic than glycerol. By 1954, however, Lovelock (1954b) was able to report successful cryoprotection of erythrocytes using a variety of other monohydric, dihydric, and polyhydric alcohols; amides; and sugars; including methanol, acetamide, and glycerol monoacetate. Dextran and PVP were added to the list of cryoprotective substances by Bricka and Bessis (1955). Lovelock & Bishop (1959) reported cryoprotection of red cells afforded by DMSO. Tullis et al (1962) reported that in June 1956, the Blood Research Laboratory was established at the U.S. Naval Hospital, Chelsea, Mass., under the auspices of the Department of Defense. Its purpose was to determine the feasibility and practicality of this method (glycerolization and low-temperature freezing) in a military hospital blood bank.

Several authors whose work has been reviewed for this study have recorded other "firsts" of interest to a historian.

> Sloviter (1953) noted the first blood transfusion reported (Lower, R.: *Philosophical Transactions* 1: 352, 1666). Huggins (1964) credits the finding that freezing and thawing caused hemolysis of human erythrocytes to Pouchet, M.F.A.: Recherches experimentales sur la congelation des animaux. *Jour. l'Anat. et Physiol.* 3: 1-36, 1866. The discovery of blood groups (Landsteiner, K.: *Wien. Klin. Wschr.* 14: 1132, 1901) is cited by Sloviter (1953). Next in chronological sequence it was recorded by Strumia, M.M. (1954) that the use of sodium citrate as an anticoagulant was introduced in 1914 (Hustin, A.: Note sur une nouvelle methode de transfusion. *Bull. Soc. Roy. Sci. Med. Natur. Bruxelles,* April, 1914); and that the addition of dextrose to citrate solution was reported (Rous, P., and Turner, J.R.: The preservation of living cells in vitro. *J. Exper. Med.* 23: 219, 1916). Huggins and Grove-Rasmussen (1965) credit the isolation and

purification of A and B substances in human blood groups to Witebsky, E., Klendshoj, W.C., and Swanson, P.: Preparation and transfusion of safe universal blood. *Jour. Amer. Med. Assn.* 116: 2654-2656, 1941. Sloviter (1953) cited the facts that Rous and Turner (op. cit.) were the first to recommend the use of preservative solution containing sodium citrate and dextrose and first showed that whole blood could be satisfactorily preserved in vitro for 2 weeks or more. Sloviter (1953) cited the first reported instance of the transfusion of preserved whole blood (Robertson, O.H.: *Brit. Med. J.* 1: 691, 1918).

Both Strumia, M.M. (1954) and Chaplin & Mollison (1954b) noted the first acidification of citrate-dextrose anticoagulant for adjustment of the pH of blood-citrate-glucose mixtures to about 7.2, which appreciably prolonged the storage life of red cells (Loutit, J.F., & Mollison, P.L.: Advantages of a disodium citrate-glucose mixture as a blood preservative. *Brit. Med. J.* 2: 744, 1943; and Loutit, J.F., Mollison, P.L., and Young, I.M.: *Quart. J. Exp. Physiol.* 32: 183, 1943). Sloviter (1953) also cited the report in 1943 that by controlling the freezing and thawing time as well as the concentration of glucose, it was possible to freeze human blood with practically no destruction of the erythrocytes (Florio, L., Stewart, M., and Mugrage, E.R.: The effect of freezing on erythrocytes. *J. Lab. Clin. Med.* 28: 1486-1490, 1943). Sloviter (1953) cited the report of the development of ACD (Gibson, S.T.: *New Eng. J. Med.* 239: 544, 1948). In 1951, Mollison and Sloviter (1951) reported that glycerolized erythrocytes could be frozen to -79°C. in relatively large quantities with relatively little lysis, and show normal in vivo survival after being thawed and deglycerolized. Sloviter (1951a) reported the finding that glycerol could be removed from erythrocytes by dialysis and the article by Mollison and Sloviter (1951) contains a sketch of a simple dialysis apparatus. Smith (1961) credits Sir Charles Harington with suggesting to Sloviter (1951a) the technique of removing glycerol gradually by dialysis.

Lovelock (1954a) credits Mollison and Sloviter (1951) in England and Ivan W. Brown, Jr., in the United States, whose earlier work is reported in Brown and Hardin (1953), with simultaneously carrying out the first successful transfusions using human erythrocytes which had been stored in the frozen state using glycerol as a preservative. Lovelock (1953,

1953a) advanced the concept that concentration of electrolytes within and without the red cell, as water was removed in the form of ice, was the most important physical change that caused damage during freezing.

Hurn (1968) credits Sloviter for originally making the suggestion in 1952 that frozen storage of glycerolized blood might be a suitable means for preserving erythrocyte antigens. Doebbler et al (1966a) noted that the action of compounds cryoprotective to erythrocytes, in terms of preventing salt concentration, retarding ice growth, and preventing freezing by the hydrogen bonding of solvent water, had been investigated by Lovelock (1953a, 1954b) and by Doebbler and Rinfret (1962), as well as by others. Meryman and Kafig (1955, 1955a) showed that erythrocytes frozen in liquid nitrogen could survive in vivo after thawing and deglycerolization. The rate of ice crystal growth as a factor in causing freezing injury to erythrocytes was suggested by Lusena and Rose (1956). In the same year, Tullis et al (1956) reported the development of a method of processing whole units of blood. The double-chromium tagging method of making survival studies was first reported by Doebbler et al (1962a). Factors involved in injury to red cells during rapid freezing have been studied by Rinfret (1963c), while Luyet et al (1963) have reviewed similar factors involved during rapid thawing.

An editorial, "Frozen Blood Banking - A New Frontier," in the *Journal of Abdominal Surgery* (1962) hails the development by Haynes and Tullis of long-term preservation of blood by freezing, using the Cohn "infractionation" method. Meryman (1966) pointed out the need for participation at the basic research level of biochemists and molecular biologists experienced in research in cell function where the effects of freezing become lethal.

The growing interest in cryobiology resulted in the formation of the Society for Cryobiology whose first annual meeting was held in Washington, D.C., August 24-26, 1964.

Chapter 2

ADDITIVES

COMPOUNDS WHICH HAVE BEEN ADDED in the processing of blood for transfusion, including preparation for storage in the frozen state, may be classified as anticoagulants, antioxidants, purine nucleosides, and both intracellular and extracellular cryoprotective agents.

Anticoagulants

In the cryopreservation of blood, an early step in the processing usually involves the use of an anticoagulant solution into which whole blood is drawn from the donor. Over the years some form of citrate solution has proved to be most satisfactory, although according to Prankerd (1961), heparin and ethylenediaminetetraacetic acid (EDTA) are suitable anticoagulants.

Heparin has antithromboplastic and antithrombic properties in that it inhibits the clotting enzymes. Other anticoagulants interfere with the catalytic action of calcium in the complex enzymatic process of clotting. From the point of view of a chemist, Lehmann, H. (1965) feels that perhaps the most important aspect of blood preservation is the choice of a suitable anticoagulant. Since its introduction in 1914, citrate has been the anticoagulant most widely used. Lehmann states that 1 g. of disodium citrate is only sufficient to prevent coagulation in 420 ml. of blood if the container is constantly agitated. In practice 2 g. are used. Chaplin et al (1954) record the assumption that citrate does not penetrate the erythrocytes. That the amount of citrate to be used varies in different formulas is indicated in the examples to be cited. Bricka and Bessis (1955) mention the use of 0.4 percent trisodium citrate. Sodium citrate and disodium citrate have also been used in combination with dextrose or glucose, with or without citric acid. The combination of acid-citrate-dextrose mixtures, known as ACD solution, is now well known. Strumia, M.M. (1954) noted that by 1947, ACD solution had become adopted generally as the standard anticoagulant and preservative.

Several formulas for anticoagulant solutions have been proposed which vary significantly in the volume of water and in the dextrose content. Examples of such variations as found in the literature are given verbatim in Table 1. An attempt to standardize the formula for ACD was made by the National Institutes of Health (NIH) of the Public Health Service (PHS), U.S. Department of Health, Education, and Welfare. Two formulas which were developed and published in 1955 are now called *NIH-A* and *NIH-B*, respectively, and will allow, after 21 days of storage at 4°C., a survival of at least 70 percent of transfused cells after 24 hours. Sproul and Sloan (1957) reported the formula (Table 1).

The anticoagulant EDTA, as pointed out by Lehmann, H. (1964), acts like citrate in binding blood calcium but is about ten times as potent as citrate for this purpose. EDTA is an excellent anticoagulant for platelets. It is not unusual to find authors reporting the use of heparinized blood without stating amounts or proportions. De Verdier et al (1965), however, reported using 20 mg. of heparin in 30 ml. of 5 percent glucose per 400 ml. of blood as an anticoagulant solution.

Antioxidants

Chaplin et al (1957, Abst. 1958) showed that when erythrocytes protected by a citrate-glycerol solution are equilibrated with oxygen prior to storage at -20°C., additional hemolysis and loss of potassium ion occur in a greater amount and at a more rapid rate than in suspensions not oxygenated. They found that such damage can be prevented by addition of an antioxidant such as glutathione or hydroquinone, or by use of a buffered suspending medium. However, the in vivo survival of red cells stored for 1 year at -20°C. in the presence of ascorbic acid (an antioxidant) and glycerol was found to be poor.

Metabolic Additives

Attempts have been made to improve the viability or survival of stored blood by addition to the anticoagulant ACD solution of such purine nucleosides as inosine, guanosine, and adenine. Strumia, M.M. (1965a), investigated the loss of adenosine triphosphate (ATP) in erythrocytes subjected to freezing and thawing. In ACD blood stored at 4°C., the loss of ATP has been one of the most constant manifestations of damage to red